Antibacterial Activity of Novel Self-Disinfecting Surface Coatings

A thesis submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

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Dedication

I dedicate this work for the soul of my father; Mr Omar Elfakhri our dream finally comes true.

To the best mum in the whole world, Mrs. Fatema Shalluf and to my wonderful dear husband, Mr Nagi Borawi for their never-ending love, encourage and support.

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Declaration

“I certify that this report consists of my own original work. All quotations from published sources are acknowledged as such in the text.

Signed:

Souad Elfakhri

List of Abbreviation

APT-FACVD	Atmospheric Pressure Thermal Flame Assisted Chemical Vapour Deposition
BS	British Standard
BSA	Bovine serum albumen
BTA	Benzotriazole
CAI	Community acquired Infections
CVD	Chemical vapour deposition
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EPS	Extracellular polymeric substances
ESBL-*E. coli*	Extended Spectrum β- lactamase Producing *Escherichia coli*
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa*, and *Enterobacter species*
FACVD	Flame Assisted Chemical Vapour Deposition
HCAIs	Health Care Acquired Infections
HPV	Hydrogen peroxide vapour
LPS	Lipopolysaccharides
MB	Microbial burden
MDRB	Multidrug resistance bacteria
MRSA	Methicillin resistant *Staphylococcus aureus*

NB	Nutrient Broth
PBP	Penicillin binding proteins
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PG	Peptidoglycan
PTPs	Protein tyrosine phosphatases
QACs	Quaternary ammonium compounds
Rh123	Rhodamine 123
R.O.S	Reactive Oxygen Species
SEM	Scanning electron microscope
TAs	Teichoic acids
T.S.A	Tryptone Soya Agar
T.S.B	Tryptone Soya Broth
VBNC	Viable But non-Culturable
VRE	Vancomycin resistant *Entrococcus faecium*
XRF	X-ray fluorescence

Abstract

The antibacterial activities of different thin films (TiO_2/CuO, Cu/SiO_2 and Ag/SiO_2) prepared by flame-assisted chemical vapour deposition (FACVD) and atmospheric pressure thermal (APT-FCVD) for TiO_2/CuO films, were investigated against standard strains of bacteria used for disinfectant testing and against multi-antibiotic resistant bacteria that have been shown to persist in the hospital environment. These included; MRSA strains (EMRSA15 and two recent clinical isolates MRSA 1595 and MRSA 1669), extended spectrum β-lactamase (ESBL) producing *Escherichia coli*, a second (ESBL- 2) producing *Escherichia coli*, KPC^+ (carbapenemase producing) *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Listeria monocytogenes*, *Salmonella enterica ser typhimurium*, and vancomycin resistant *Enterococcus faecium* (VRE) . The Antimicrobial activity of the above coatings (Cu/SiO_2 and Ag/SiO_2) was investigated based on the BS ISO 22196:2009 and 2011 Plastics – Measurement of antibacterial activity on plastics and other porous surfaces. The activity of TiO_2/CuO films was investigated based on the BS ISO 27447:2009 Test method for antibacterial activity of semiconducting photocatalytic materials.

On the TiO_2/CuO films, the bacteria were killed by UVA irradiation of the photocatalyst with a >5 log kill within 4-6 h except for the MRSA where a 2.3 log kill was obtained after 6 h increasing to >5 log after 24 h. There was antimicrobial activity in the dark which was enhanced by irradiation with fluorescent light. There was also activity at 5ºC under UVA but activity was lower when fluorescent light was used for illumination. The Cu/SiO_2 coating showed a >5 log reduction in viability after 4 h for the disinfectant test strain (*E.coli*) and for some pathogenic strains include; *Acinetobacter baumannii, Klebsiella pneumoniae* and *Stenotrophomonas maltophilia*. However, their activity against the other hospital isolates was slower but still gave a >5 log reduction for extended spectrum β-lactamase producing

Escherichia coli and *Salmonella enterica typhimurium,* and > 2.5 log reduction for vancomycin resistant *Enterococcus faecium*, *Listeria* and methicillin resistant *Staphylococcus aureus* within 24 h. The coating was also active at 5ºC but was slow compared to room temperature. The highest activity of copper /silica films was seen at 35ºC, but bacterial cells were also killed on the control surfaces. The Ag/SiO_2 coating was also active against pathogenic bacteria; however the coating was not hard or durable as other coatings used. The activity on natural contamination in an in use test in a toilet facility was also determined for coated ceramic tiles (Cu/SiO_2 and Ag/SiO_2) and coated steel. The results demonstrated that the tiles were highly active for the first 4 months period and the contamination was reduced by >99.9%. However, tiles lost some of their activity after simulated ageing and washing cycles. The Cu/SiO_2 coated ceramic tiles placed in Manchester Royal Infirmary also showed antimicrobial activity and no indicator organisms were detected.

The coatings had a good activity against both standard test strains and clinical isolates. The coatings (copper surfaces in particular), may have applications in health care by maintaining a background antimicrobial activity between standard cleaning and disinfection regimes. They may also have applications in other areas where reduction in microbial environmental contamination is important, for example, in the food industry. However, the optimum composition for use needs to be a balance between activity and durability.

Keywords: TiO_2, CuO, Ag, Antimicrobial; Chemical vapour deposition; Copper; Disinfection surface; Pathogenic bacteria (hospital pathogen).

1. Introduction

1.1 General Introduction

Due to the continual threat to human life, killing or controlling the growth of pathogenic micro-organisms such as bacteria, viruses and fungi on inanimate surfaces continues to be a major concern around the globe. Antimicrobial agents have been used for many years to overcome pathogenic organisms in a wide range of applications (in hospitals, the home and industrial premises). However, using them for a long time has led to the development of resistant microbes (Maillard, 2002, Maillard, 2005). Vegetative bacteria are more sensitive to chemical agents than bacterial spores. However, the number of antibiotic resistant bacteria has increased in recent years due to the overuse of antibiotics and biocides which in many cases have led to the development of cross resistance. Therefore, new, safe and effective biocides are continually needed to overcome problems associated with micro-organism adaptation and the development of resistant strains (Hamouda and Baker, 2000).

Microbes are associated with most living forms on earth, including human beings, and in many cases are essential for the survival of their host. Thus it is not always useful to kill them by disinfecting our surroundings. Only areas where the adhesion of microbes is unwelcome should be sterilized or disinfected. Such antimicrobial surface modifications are needed in the medical field and in material sciences (Bieser *et al*., 2011).

The ability of bacteria to grow on different surfaces is causing huge concern in hospitals and food industries due to the increased risk of bacterial infection. The bacterial contamination of hospital

surfaces (such as patient rooms, nurse stations and kitchens) and food preparation surfaces (including refrigerators) has been extensively reported (Champagne and Helfritch, 2013).

Many microorganisms are able to survive for prolonged periods on inanimate surfaces and cause bio-contamination. This can lead to transmission of infectious diseases, especially in hospital settings (nosocomial or health care acquired infections-HCAI). Such infections are caused by several pathogens, including methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant Enterococci (VRE), and *Acinetobacter baumannii*, (Otter *et al*., 2011). For example, MRSA by itself caused about 8% of all hospital acquired infections in the U.S.A. in 2010 (Nie *et al*., 2010). Patients with compromised immune systems are at high risk of these infections which, in some cases, leads to prolonged hospital stay or possibly death (Ducel *et al*., 2002). Hospital acquired infections caused 5,000 deaths per year in England (Katsikogianni and Missirlis, 2004), and cause 17500-70000 annual deaths in the USA. These infections cost the UK's National Health Service one billion pounds each year which was the equivalent of 1% of the total national hospital budget and between 17 and 25 billion dollars added to health costs every year in the USA (Schabrun and Chipchase, 2006).

Contamination of surfaces in a hospital environment, such as on medical devices, causes about 45% of hospital acquired infections. Surgical equipment, medical staff and resident microbiota on patients' skin are other infection sources that cause about 55% of HCAIs (Madkour *et al*., 2008). According to French standards, equipment containing 5 cfu/cm^2 or more micro-organisms should be considered as contaminated surfaces. In hospitals, the level of equipment contamination was found to be four times higher than the level of French standards. This high level of contamination may be due to failure in infection control, mainly in terms of hand washing and cleaning of equipment and to overuse of antibiotics, which lead to increased microbial resistance. It has been reported that more

than one-third of infections acquired in a healthcare setting could be prevented through hand washing and adequate cleaning of equipment (Schabrun and Chipchase, 2006).

Cleaning is a key factor to overcoming these infections. The use of disinfectants and bactericidal surfaces together could potentially reduce the incidence of infectious diseases (Warnes *et al*., 2010). The antimicrobial activity of TiO_2 has been known for years (Matsunaga *et al*., 1985) and a wide range of micro-organisms have been shown to be susceptible (Foster *et al*., 2010) However, although TiO_2 coated materials have been widely used for their self-cleaning activities, there has been no widespread uptake for antimicrobial use in the healthcare sector despite its potential (Page *et al*., 2009). Metals that exhibit antimicrobial properties, such as copper and silver, have been used for clinical and non-clinical purposes for centuries due to their ability in limiting the growth of a wide spectrum of micro-organisms (Gudipaty *et al*., 2012). In contrast to other metals, silver is highly toxic to micro-organisms compared to low toxicity to mammalian cells (Li *et al*., 2010). The production of biocide-coated surfaces (thin coated films) is based on coating solid surfaces with a thin film of metal such as copper, silver or titanium, applied by different techniques (Foster *et al*., 2010). Biocide releasing surfaces that depend on diffusible ions such as copper and silver do not rely on light for their antimicrobial activity unlike TiO_2 which requires UVA illumination (Page *et al.,* 2009).

The beneficial effect of copper for humans has been known for at least 4,000 years (Efstathiou, 2011). Copper workers were immune to cholera in 1832 in Paris during an outbreak. Thus copper began to be used in medicine in the 19th and early 20th centuries and it continued to be used in medicine until antibiotics become commercially available in 1932 (Dollwet., *et al* 1985). Despite this fact, it seems its usefulness has been forgotten. However, due to the findings of a recent investigation into the effectiveness of copper against a wide range of micro-organisms has led to a

re-introduction of this metal in healthcare (Efstathiou, 2011). Biocide surfaces composed of copper or copper alloys are now recommended to be used for surfaces that are in contact with human skin and food. This can possibly be achieved by using solid copper or copper alloy equipment or by copper coating surfaces. However, due to cost of solid copper, copper coated surfaces may be preferred (Champagne and Helfritch, 2013). The clinical trials which appeared after more than ten years of laboratory research confirmed the benefit of copper as an active tool in reducing the environmental bio-burden in a number of healthcare settings globally. The first fully qualitative clinical trial in the UK was in 2007 at Selly Oak Hospital in Birmingham (Efstathiou, 2011). Copper was also used in other applications, areas such as transport and schools where a high population of people were usually present. Results from an elementary private school in Athens, Greece, with 2,500 students, showed that the surfaces that had been replaced with surfaces made of copper alloys gave 90–100 % less contamination compared to other non-copper surfaces (Efstathiou, 2011).
The importance of environmental contamination plays in disease transmission for HCAIs has been known for many years but that antimicrobial surfaces can help to reduce transmission has only recently been confirmed (Schmidt *et al*., 2013). This role will be surveyed in the following sections together with the potential for Cu, Ag and TiO_2 surfaces to prevent such infections.

1.2 Nosocomial infections

Nosocomial infections or healthcare associated infections (HCAIs) have been known of for several decades; since patient hospitalisation began (Swartz, 1994). However, infecting micro-organism species and their properties have changed over time. A study at Boston City Hospital in 1946 showed that 90% of *Staphylococcus aureus* strains were susceptible to penicillin (Finland, 1955). A study in 1970s showed that 75% of *S. aureus* strains were penicillin resistant, while more recently more than 95% strains were penicillin resistant (Neu, 1992). HCAIs are the major cause of death and have led to increased morbidity on the part of hospitalized patients. Basically, infections that occur within 48 h of admission or stay at a healthcare facility and that were not present or incubating at the time of admission, are commonly considered nosocomial infection (Kelly and Monson, 2012). In addition, infections that occur after 3 d of discharge or 30 d of an operation are also considered nosocomial infection. These infections affect approx.1 in 10 patients admitted to hospital (Inweregbu *et al*., 2005). There are many different definitions of HCAIs in the scientific literature which help to differentiate such infection from community acquired infection (Filetoth, 2003).

An infection which occurs before 48 h of admission is considered as a community acquired infection (CAI). The 48 h is the average time required by bacteria in a human host to develop from initial infection stage to a detection stage from a positive diagnostic test. In contrast to HCAIs, CAI are usually caused by virulent pathogens able to infect healthy people, while HCAIs are caused by organisms developed among ill patients (Henderson *et al*., 2013). Pathogenic organisms that cause nosocomial infections can be transmitted to the community in a number of different ways and particularly through health staff, visitors and discharged patients. However, the spread and development of nosocomial infections are influenced by many factors which include: 1- The characteristics of the microorganisms, including their level of antibiotic resistance, innate virulence,

and amount (inoculum) of microbial agent. 2- Patient susceptibility - the elderly and neonates are more susceptible and have lower immune status as are those with trauma or who have surgery. These are associated with a decreased resistance to infection. 3- Environmental factors (Ducel *et al*., 2002).

1.2.1 Nosocomial infection sites

Theoretically, any pathogenic organism or opportunistic pathogen may cause HCAIs if the conditions are appropriate. These conditions include a host such as a human or inanimate source and a successful transmission within the health care facilities. However, the microbes causing HCAIs are distributed unequally, and infection transmission also depends on other factors including environmental survival and transmission dynamics (Filetoth, 2008). The most frequently contaminated surfaces in the hospitals are floors, bed frames, tables, patients' clothes, pillows and mattresses (Talon, 1999). However, the level of contamination has been shown to vary depending on the body sites at which patients are colonized or infected (Boyce, 2007) and the contamination that occurred in infected patients is higher than in colonized patients (Talon, 1999). Urinary tract infection is the most common nosocomial infection which is associated with low morbidity compared with other nosocomial infections. However, it can lead to bacteraemia and death (Ducel *et al*., 2002). A summary of the distribution of the sites of nosocomial infections is shown in Figure 1.

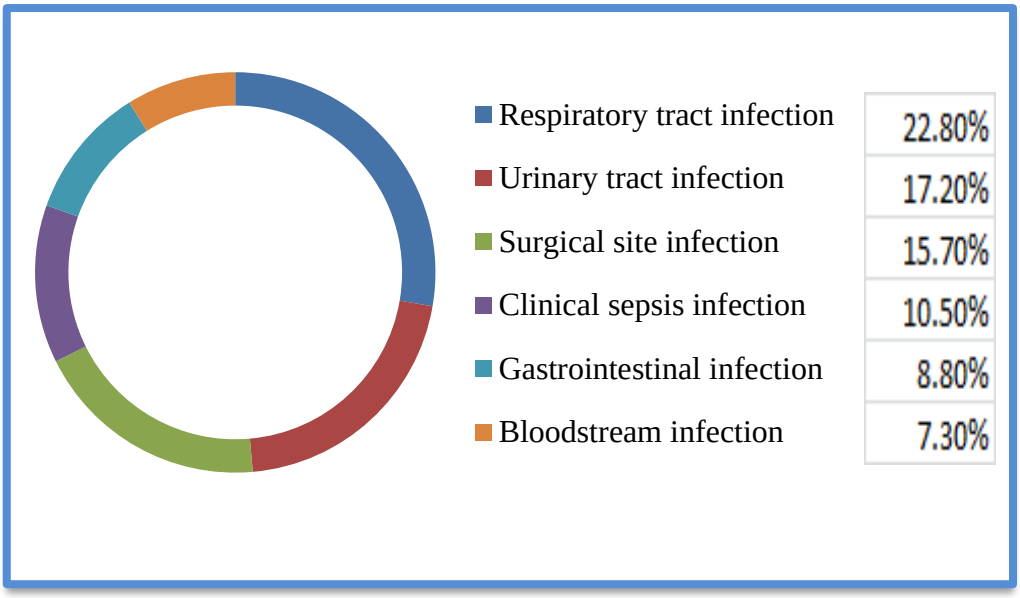

Figure 1 Sites of the most common HCAIs. Modified from Health protection agency (2011).

A study by Boyce *et al*. (1997) demonstrated that 36% of surfaces swabbed in the rooms of MRSA patients with urinary tract infections were contaminated with MRSA, compared to 6% of surfaces in the rooms of MRSA positive patients with the infection at other body sites.

In another study, ten different high-touch surfaces were swabbed in the rooms of eight gastrointestinal patients colonized by MRSA and who were suffering from concomitant diarrhoea, and in the rooms of six patients with MRSA at other body sites, but not in their stools (controls). It was found that 59% of surfaces were contaminated by MRSA in gastrointestinal and concomitant diarrhoea patients' rooms, and only 23% of surfaces were contaminated in the control rooms (Boyce *et al*., 2007). Therefore, evaluation of terminal cleaning and disinfection, which is usually performed after patients have been discharged, is highly needed (Boyce *et al*., 2011). White coats of medical workers were also reported as a source of contamination, which may play a role in transmission of pathogenic bacteria in the hospital environment, despite the fact that they are worn to protect the workers from such contamination. It has been reported that *S. aureus* was isolated from nurses'

uniforms and doctors' white coats, especially from those within the surgical specialisms (Loh *et al*., 2000). Moreover, medical students which have the most contact with patients, but who have the least knowledge about the effect of nosocomial infections, may have an increasing effect on the spread of pathogenic organisms. It has been shown in a survey study that medical students do not wash their white coats routinely and many of them wash their coats only if they are stained or visibly dirty. Results of bacteriological tests on 100 white coats from medical students showed that all the coats were contaminated, but by different degrees. The highest level of contamination was present on the coats' sleeves and pockets, since the sleeve of the coat is the site that most frequently comes into contact with patients when students examine patients. This is despite the fact that over a third of medical students laundered their coats monthly. Therefore, rather than terminating the use of the white coat, different material and a change of washing regimes may be applicable (Loh *et al*., 2000).

1.2.2 Microorganisms causing HCAIs

HCAIs are caused by different pathogens which include bacteria, viruses and fungi (Nguyen, 2007). Bacterial and fungal infections are less common compared to viral infections during the winter. However, they are significantly associated with higher morbidity and mortality (Nguyen, 2007). In fact, describing the importance of the problem accorded to the antimicrobial-resistant pathogens is difficult, because the levels of antimicrobial resistance that cause HCAIs vary among patient populations, different types of healthcare facilities and for different geographic areas (different countries). However, the findings from such attempts may help infection control and public health communities to find the appropriate solution for this problem more efficiently (Hidron *et al*., 2008). Rates of mortality and morbidity associated with multidrug resistant bacteria are increasing daily in both community and hospital settings. The term `bacterial resistance' refers to strains that are not

affected (inhibited or killed) by a concentration of antibiotic which most strains of bacterial cells are susceptible to *in vivo* and can be attained in e.g. blood or urine.

Two categories bacterial resistance to antibiotics are intrinsic and acquired resistance. Intrinsic resistance is an innate property of a bacterial cell and usually refers to naturally low membrane permeability and absence of an enzyme-metabolic pathway. Acquired resistance, which is a cause for concern in the clinical setting, usually arises by mutation, the acquisition of plasmids or by transformation or transduction. Bacteria are able to resist antibiotics by multiple mechanisms; change in target site; alteration of antibiotic; decreased antibiotic accumulation or active efflux of antibiotic from the cell; and overproduction of target site. Resistance can be a result of a single mechanism or combined expression of more than one mechanism (Russell, 2000).

The term multidrug resistance (MDR) is used to describe bacteria that are able to resist one or more antibiotics in three or more antibiotic classes, while bacterial strains that are resistant to all antibiotics are termed as extreme drug resistant strains (Bassetti *et al*., 2013). Moreover, the term ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa,* and *Enterobacter* species) refers to a group of pathogenic bacteria that are resistant to most available types of antibiotics and have caused the majority of HCAIs during the 21st century. Despite the increased number of bacteria resistant to the antibiotics there is no increase in the discovery of new antibiotics. There has been no new class of antibiotics since 1962 (Xu *et al*., 2014). The main factors that lead to microbial resistance and the spread of MDR organisms are thought to be the misuse of antibiotics by healthcare workers for both outpatient (physicians) and hospitalized patients, poor drug quality, poor hygienic conditions (such as hand washing and proper isolation of patients with resistant infections) and the use of antibiotics in animals and in the agriculture industry, such as in food production. Antibiotic resistance is a global problem, but poor and developing nations are more affected due to the fact that antibiotics can be

obtained easily over the counter without doctors' prescriptions, whereas in the developed countries access to antibiotics is much more limited (Alanis, 2005).

1.2.2.1 Multidrug resistant bacteria used in this study

1.2.2.1.1 Hospital related pathogens

S. aureus

S. aureus is a facultative anaerobic bacterium which is known as part of normal human microbiota, but can also act as a virulent pathogen (Kelly and Monson, 2012). *S. aureus* produces several proteins helping it to cause infection. These proteins allow it to adhere to a host surface and avoid detection by the host's defences. These include an antiphagocytic capsule and protein A. In addition, *S. aureus* is also able to secrete chemotaxis inhibitory proteins and many other proteins. Despite the fact that MRSA can be transmitted directly through contact with infected or colonized individuals or/and through their environment, MRSA can colonize parts of healthy people's bodies (skin or nares) for weeks or even years without progression to active infection (Kelly and Monson, 2012). *S. aureus* are able to resistant penicillin by producing a specific enzyme called staphylococcal penicillinase. In addition, MRSA also become resistant to modified types of penicillin named methicillin or oxacillin which were resistant to the action of the staphylococcus penicillinase. The action of methicillin was based on the blocking of the penicillin binding proteins (PBPs), which are used by *S. aureus* to synthesise peptidoglycan in the cell wall. However, *S. aureus* acquired a new protein (PBP2a) which was not affected or blocked by methicillin. Due to this protein, nowadays MRSA is known to be resistant to all β-lactam antibiotics, including synthetic penicillins, cephalosporins and carbapenems (Pantosti and Venditti, 2009).

Vancomycin resistanct *Enterococcus*

Vancomycin is an antibiotic that can be used to treat infections caused by *Enterococcus spp* and other species such as *S. aureus*. However, some *Enterococcus* species, which are known as vancomycin-resistant *Enterococcus* (VRE, sometimes referred to as glycopeptide resistant enterococci, GRE), are no longer killed by vancomycin. The first occurrence of vancomycin-resistant enterococci was in the mid-1980s in both Europe and the United States (Leclercq *et al*., 1988). The most common infections caused by enterococci are urinary tract infections, intra-abdominal and surgical wound infections and bacteraemia (Moellering, 1998). *Enterococcus faecium* is the fourth most common pathogen to cause nosocomial infection in the world (Werner *et al*., 2008). It is a facultative anaerobic coccus and has the ability to survive under different environmental conditions. The ability of *Enterococcus faecium* to resist vancomycin is due to acquired *Van A*, B, D, E, G, L resistance genes. These genes alter the vancomycin binding site in the cell wall. In addition, these genes can be transferred to other enterococci through plasmids (Kelly and Monson, 2012).
In the past researchers had concentrated on multi drug resistant Gram-positive bacteria, particularly MRSA and VRE spp. More recently, microbiologists increasingly accept that the multidrug resistant Gram-negative bacteria cause the greatest risk to public health due to the rapid transfer of mobile resistance genes on plasmids that have the ability to spread through Gram-negative bacterial populations (Carattoli, 2009). In addition, current drug discovery programmes show little promise for the development of truly novel therapeutic drugs in the next 10-20 years (Kumarasamy *et al*., 2010).

ESBL producing bacteria

The spread of multi drug resistance MRSA in the last decades has been recently paralleled by the community infections caused by Gram-negative bacteria that produce extended-spectrum β

lactamases (ESBLs). Since the 1990s, multidrug resistant *Enterobacteriaceae* have become an important cause of urinary tract and blood stream infections within the community setting (Steindl *et al*., 2012).ESBLs are mainly produced by *Klebsiella pneumoniae* and *Escherichia coli* which cause the majority of Gram-negative HCAIs. Of particular concern are the carbapenamase producers (Carbapenamase-resistant *Enterobacteriaceae*- CRE) that render carbapenem (often referred to as the last line of defence) in-effective (Ref). ESBLs inactivate β-lactam antibiotics by hydrolysis before they reach the penicillin-binding proteins. These enzymes are also produced by non-fermentative Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Falagas and Karageorgopoulos, 2009).

A. baumannii

The importance of *Acinetobacter spp* as pathogenic bacteria has increased within the past two decades due to their ability to develop multiple resistances to the major antibiotic classes. *A. baumannii* is the most important of *Acinetobacter spp* in both causing infections and the resistance to drugs. It is a non-motile, strict aerobic coccobacillus and commonly present in soil, water, sewage and in healthcare settings. The ability of *A. baumannii* to develop multiple resistance mechanisms against the majority of antibiotics is due to its ability to produce a wide variety of enzymes hydrolysing different antibiotics including penicillins and cephalosporins (Bergogne-Bérézin *et al*., 2008).

Stenotrophomonas maltophilia

S. maltophilia is a motile, non-fermentative, aerobic Gram-negative bacillus (Senol, 2004). *S. maltophilia* is the only species of the genus *Stenotrophomonas* known to cause infection in humans (Looney *et al*., 2009). In the past, *S. maltophilia* was associated with limited pathogenicity; however recent reports indicate that infection with this organism in severely compromised patients

(hospitalized patient in particular) is associated with significant morbidity and mortality. Bacteraemia and pneumonia are among severe infections that are associated with high mortality, whereas wound and urinary tract infections are less frequently caused (Senol, 2004). *S. maltophilia* is found mainly in any aquatic or humid environment, including the drinking water supply and in many other sources such as soil, plants, raw milk and in human and animal faeces. In addition, *S. maltophilia* is also able to survive on medical devices as well as colonise respiratory tract epithelial cells which led to its emergence as a major nosocomial pathogen especially in the ICU setting (Looney *et al*., 2009). Treatment of *S. maltophilia* infections is complicated due to its resistance to many of the currently available broad-spectrum agents including carbapenems. The antibiotic resistance of *S. maltophilia* is referring to multiple mechanisms; including a multidrug efflux system and outer membrane impermeability (Senol, 2004).

1.2.2.1.2 Foodborne Pathogens

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive motile bacterium and a facultative intracellular parasite. It has the ability to grow under various environmental conditions such as high concentration of salt, pH (Yan *et al*., 2010), and temperatures between -0.4 and 50°C (Walsh *et al*., 2001). It is mainly a foodborne pathogen which is found everywhere in the environment and able to cause listeriosis in humans and animals. *Listeria monocytogenes* is the cause of a number of severe infections in humans, such as septicaemia, meningitis, gastroenteritis, etc. *Listeria monocytogenes* is usually susceptible to a wide range of antibiotics which proved to be active against Gram-positive bacteria (Yan *et al*., 2010). However, after the first isolation of a multi-resistant strain in France in 1988, other resistant strains have been isolated from different sources including food, environment and human listeriosis. β-lactam antibiotic (e.g. penicillin or ampicillin) is the common current treatment of listeria infection, alone or in combination with an aminoglycoside (e.g. gentamicin) in the case of

immunocompromised patients (Ref). Despite the fact that many antibiotic-resistant bacteria in foods are saprophytic or commensal; their resistance genes can be transferred through plasmids to other foodborne bacteria, including *L. monocytogenes* within the gastrointestinal tract. Enterococci and streptococci are the common source of *L. monocytogenes* resistance genes (Conter *et al*., 2009).

Salmonella enterica serotype Typhimurium

Salmonella enterica is one of the most important foodborne pathogens worldwide; it affects over one billion humans and results in around three million deaths every year (Karatzas *et al*., 2008). Severe human *Salmonella* infections are commonly treated by fluoroquinolones and third-generation cephalosporins. However, resistance to these and other antimicrobial drugs, as well as multidrug resistance, has increased over the last several decades (Wright *et al*., 2005). This resistance is thought to be due to the intensive use of antibiotics in veterinary, clinical and growth promotion in farm animals (Karatzas *et al*., 2008). This facilitates the transmission to humans mainly by ingesting food; meat in particular, dairy products and other foods contaminated by animal faeces or by cross-contamination from foods contaminated with *Salmonella* species (Wright *et al*., 2005). *Salmonella enterica* is a new name proposed as a replacement for the name "*Salmonella choleraesuis*." The later name was used to refer to a single species of *Salmonella* which has 2500 different serotypes or serovars (based on DNA hybridization), and have familiar names (e.g., *Salmonella* serotypes Typhimurium,). The species name *S. choleraesuis* is confusing, because there is another serotype of Salmonella known as *Salmonella* serotype *Choleraesuis*, which is associated with bacteraemia. According to the new nomenclature system, "*Salmonella typhimurium*" would be renamed as "*Salmonella enterica* serotype *Typhimurium*." Even though this nomenclature system is not adopted formally by the International Committee of Systematic Bacteriology, this system has been accepted for use by the World Health Organization and in publications of the American Society for Microbiology (Acheson and Hohmann, 2001).

1.3 Biocides

One way of reducing environmental contamination by microorganisms is the use of biocides. Biocides are chemical products which have been produced to act as poisons or inhibitory agents against a wide range of organisms (Kähkönen and Nordström, 2008) and have been used for centuries to tackle pathogenic organisms. The use of biocides is high in the healthcare environment, mainly for the disinfection of surfaces, water, equipment, and skin antisepsis, and also for the sterilization of medical devices and the preservation of pharmaceutical and medical products (Maillard, 2005).The increased use of biocides has led to increased organism resistance, therefore development of new biocides is needed. The microbial resistance to biocides differs, not only between different types of micro-organisms, but also between different strains of the same species (Maillard, 2002). The biocide resistance of bacteria was first recognized in 1936 by Heathman and others who discovered the resistance of *Salmonella typhi* to chlorine. In contrast, the links between biocide resistance and antibiotic resistance of micro-organisms have only been recognized more recently (Fraise, 2002). The possible reason for links between biocide and antibiotic resistance is that the resistance genes to both can sometimes be found on the same plasmid (Sidhu *et al*., 2002). The exact resistance mechanism of micro-organisms to biocides is still unclear , and even though the action of antibiotic and biocides are different (antibiotics interact very specifically whereas biocides may have many different targets in the cells) many common mechanisms that bacteria use for antibiotic resistance is also reported for biocides resistance (Davin-Regli, 2012). Bacteria can become resistant by pumping out biocides via efflux systems or make their membrane less permeable to them, may inactivate them by producing detoxifying enzymes which render biocides ineffective, or by modifying some parts of their structure that biocides attack. However, the latest mechanism is not largely counted in biocides resistances, since there are many different sites that biocides can attack (Fraise, 2002). Moreover, bacteria that have been previously controlled by a

biocide can develop resistance by acquiring resistance genes. Langsrud *et al*. (2003) showed increased resistance of *Pseudomonas spp* pre-adapted with quaternary ammonium compounds (benzalkonium chloride and didecyl dimethylammonium chloride) which was due to the phenotype changes. The resistance mechanism involved in the adaption process was thought to be multifactorial rather than having only one factor, since the changes were seen from the first subculture. It is well documented that some pathogenic bacteria present in biofilms form on hospital surfaces (see below) and, in contrast to planktonic populations of bacteria, biofilms have reduced susceptibilities to biocides and antibiotics. In a recent study, authors showed that none of the biocides (benzalkonium chloride, chlorhexidine gluconate and triclosan) commonly used in hospital were able to kill 100% of MRSA and *P. aeruginosa* biofilms. Therefore, the use of biocides in hospital sectors is not sufficient to eliminate bacterial biofilms (Smith and Hunter, 2008). Even though the number of products needing new biocides is increasing, the number of available biocides is decreasing. This was due to the high costs related to registration of new chemical substances under the current regulation. Four million euros has been estimated as an approximate registration cost of an active substance in accordance with the Biocidal Product Directive (BPD) European Union 98/8/EC (Kähkönen and Nordström, 2008).

Based on their use, chemical biocides fall into two broad groups: antiseptics and disinfectants. The term antiseptic refers to substances which are used to eliminate micro-organisms from the skin or mucous membranes and there are many different products used in health care. These include alcohols, chlorhexidine, chloroxylenol, iodine and iodophors, quaternary ammonium compounds (QACs) and triclosan (Weber *et al*., 2007), whereas disinfectants are used for inanimate surfaces. Some of the antiseptic compounds are also used for both disinfection and preservation depending on the concentration (Maillard, 2005). For example; alcohols are added to cosmetic products to protect

them from microbial invasion. It is also used on surfaces to be disinfected in the food industry and hospitals. Moreover, it has been shown that a low concentration of ethanol, ranging from 0 to 15%, enhanced the sterilization effects of high-pressure thermal sterilization (combination of moderate temperature and very high pressure), used against *Bacillus subtilis* spores in keeping the nature of food. This might be due to the effects of ethanol on proteins (Zhang *et al.*, 2012b). Alcohols have a broad spectrum of activity against vegetative cells of a variety of micro-organisms, including mycoplasma, but have no effect on spores, protozoan oocysts and certain non-enveloped viruses (Weber *et al*., 2007). The alcohols compounds most used for antiseptic purposes are ethyl alcohol (ethanol), methyl alcohols (methanol) and isopropyl alcohol (isopropanol). The concentration of 60-95% is generally used for antiseptic purposes but low concentration is recommended when used as preservatives in pharmaceutical and cosmetic products. One disadvantage of alcohols is that their efficacy is short-lived due to its rapid evaporation (Barah, 2013). QACs are widely known as useful antiseptics and have a broad spectrum of activity with the exception of endospores and are bacteriostatic to mycoplasmas (Barah, 2013). They are also used for disinfection of non-critical hard surfaces and domestic cleaning products (McBain *et al*., 2004).

1.3.1 Use of biocides in prevention of HCAI

It is well documented that hand hygiene is one of the key elements that effect the infection prevention and control in healthcare facilities, since multi resistant organisms, such as MRSA, are widely known to be transmitted between patients through their hands (Horner *et al*., 2012). Chlorhexidine is most commonly used in antiseptic products, mainly in hand washing (for more than 50 years) and it is less likely to cause dry skin. This is due to its long lasting efficacy against micro-organisms, Gram-positive in particular, safe and lack of systemic side effects (Horner *et al*., 2012). A hand wash of chlorhexidine based soap had little effect on resident hand microbiota, whereas on

transient hand microbiota, bacteria reduced by 2.1 to 3 $\log_{10}$ units(Kampf and Kramer, 2004). Even though chlorhexidine is a powerful biocide, its activity is pH dependent and is greatly affected in the presence of organic matter such as blood and pus (Barah, 2013).

Despite the fact that antiseptic and disinfectant compounds are very active against a wide variety of organisms some antiseptics were involved in a number of outbreaks, which was due to the use of contaminated antiseptics. The outbreaks following the use of antiseptics were higher compared to those following the use of disinfectant. Most antiseptics are contaminated due to user error rather than microbial contamination. User error includes the use of over diluted solutions, outdated products, tap water to dilute products rather than sterilised water and the incorrect selection of a relevant product (Weber *et al*., 2007). Moreover, the outbreaks associated with contaminated high level disinfectant (kills all types of micro-organisms except high population of bacterial spores) are rare, whereas outbreaks of contaminated intermediate (kills most of micro-organisms with the exception of some fungi and no effect on spores) and low disinfectant (has no effect on mycoplasma and spores) are more common. And the most likely organism to cause outbreaks associated with contaminated biocides are Gram-negative bacteria which may be due to their complex structure of cell well (Weber *et al*., 2007). In general, even though disinfectant kills most vegetative bacteria its action is temporary because it cannot tolerate environmental aggregation such as contact with water and wiping (De Lorenzi *et al*., 2013).

1.4. Role of the healthcare environment in transmission of HCAIs and surface contamination

Environmental contamination has been recognised as playing a role in the transmission of HCAIs (Weinstein and Hota, 2004) . The importance of the environment in the transmission of HCAIs has been documented for *Acinetobacter baumannii* (Aygün *et al*., 2002, Wagenvoort and Joosten, 2002),

. MRSA (Rampling *et al*., 2001), and vancomycin resistant enterococci (Martinez *et al*., 2003). However, it is only recently that the hospital environment has been recognised as an important reservoir for the transmission of such infections (Weber and Rutala, 2001, Bartley and Olmsted, 2008, Dancer, 2009). Many factors can facilitate the environmental transmission of such microbial contaminates. These include the ability of the pathogen to survive for prolonged periods and maintain their virulence on environmental surfaces and to transiently colonize both patients and health care workers (Weber *et al*., 2010).

In hospitals, patients are more likely to acquire HCAIs if the previous occupant of the room had such an infection (Boyce *et al*., 1997, Drees *et al*., 2008, Nseir *et al*., 2011, Shaughnessy *et al*., 2011). It has been known for many years that the environment around a patient becomes contaminated with pathogens and that disinfection only transiently reduced this (Ayliffe *et al*., 1967). Although early studies showed that routine cleaning was as effective as routine disinfection (French *et al*., 2004) and that routine disinfection had no effect on infection rates (Hidron *et al*., 2008),recent studies are contradictory. Some reports show that enhanced cleaning and disinfection of the hospital environment does indeed reduce rates of infection (Brandi *et al*., 1989, Dancer *et al*., 2009, Carling *et al*., 2010). However, surfaces can rapidly become re-contaminated even after "deep cleaning" (Hardy *et al*., 2007). Another study conducted on an intensive care unit showed that hydrogen peroxide vapour (HPV) was not an effective method of maintaining low levels of environmental contamination caused by MRSA in an open-plan environment such as intensive care unit due to rapid rate of recontamination. MRSA was isolated from 11.2% of environmental sites during the period of study (3 months) of using HPV and according to epidemiological finding; the types of MRSA present in the environment were similar to those colonising patients. In detail, MRSA was isolated from five sites (17.2%) after patient discharge and terminal cleaning using conversion cleaning procedure, whereas after HPV decontamination but before the re-admission of patients, MRSA was

not isolated from the environment. However, after re-admitting patients, the MRSA was isolated from five sites after 24 h. In fact, MRSA was isolated from 16.3% of sites after eight weeks of using HPV (results were based on sampling the environment weekly (Hardy *et al*., 2007).

The contamination of surfaces basically starts with the fundamental adherence of a very small number of microbes which, under appropriate conditions, may develop into a biofilm within 24 h (Figure 2). Biofilms primarily form when there is sufficient moisture (Hetrick and Schoenfisch, 2006). These biofilms are characterized as mono- or multi-species communities and are able to attach themselves to surfaces (bio or non-bio surfaces) and are kept together by self-produced extracellular polymeric substances (EPS). The EPS contain polysaccharides, proteins and DNA originating from the microbes. The EPS are important since they provide structural stability and enhance the intrinsic resistance of bacteria cells by the inactivation of antimicrobial agents(Høiby *et al*., 2010). Other biofilms' resistance to antimicrobial agents can be through the overexpression of stress-responsive genes, and the differentiation of a subpopulation of biofilm cells into resistant dormant cells (Kostenko *et al*., 2010).

Bacterial adhesion to a material surface is composed of two phases. Phase one starts with reversible adhesion of a cellular organism with a surface; phase two starts 2-3 h later and is identified by durable adhesion between the bacteria and the surface material as a result of specific chemical reactions between compounds on the cell and substrate surfaces which leads to irreversible molecular bridging (Hetrick and Schoenfisch, 2006, Katsikogianni and Missirlis 2004).

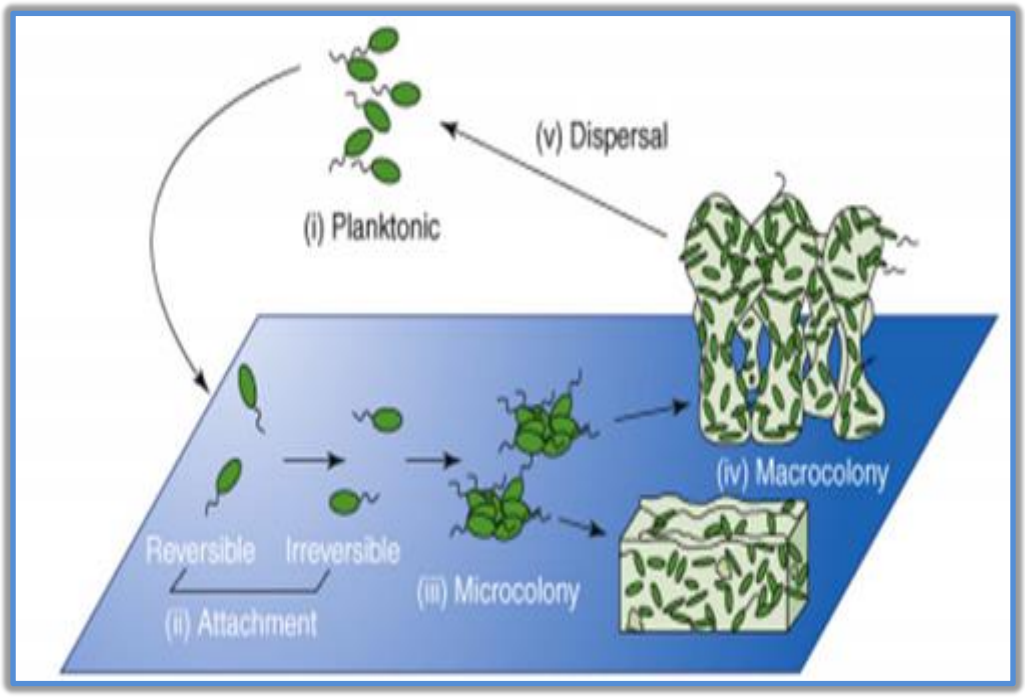

Figure 2 Bacterial biofilm formation. (Monds and O'Toole, 2009).

(i) Planktonic, (ii) Attachment. The attachment phase has often been further divided into weak stage 'reversible' and strong and durable 'irreversible' stage. (iii) Microcolony formation is the formation of discrete cell clusters, which can form by the growth of attached cells or by active translocation of cells across the surface. (iv) Microcolonies grow in size and combine together to form macrocolonies. The macrocolony shape could be a mushroom-like towers or flat structures, and cells are held within the macrocolony by an EPS matrix. Finally, macrocolonies can dissolve, and releasing planktonic cells from the biofilm giving a realistic picture of a complete biofilm formation cycle (Monds and O'Toole, 2009).

Bacteria on dry copper are not able to produce biofilms due to rapid killing by copper. On the other hand, the contaminations of public facilities such as bathrooms are usually associated with moisture and water (Nie *et al*., 2010). The water is an essential factor for both bacterial growth and biofilm formation. Therefore, identifying the nature of an organism and its surrounding environments is

among the important factors that may help to determine the ability of the organism to present on the surfaces (Fuster-Valls., *et al* 2008).

The physical characteristics of hard surfaces such as the degree of roughness or smoothness are one of a number of factors that affect adhesion and the survival of micro-organisms. Bacteria can grow and survive longer on rough surfaces than on smooth surfaces due to the increase in adherence sites and surface distances (Makison and Swan, 2006).

A study on the effect of different types of surface that are commonly used in a hospital environment including painted wood, varnished wood, Formica, standard vinyl flooring and safety vinyl flooring, on the survival of MRSA showed the greatest decline in the number of colony forming units on Formica and safety vinyl, and the lowest decline rate on painted and varnished wood as a result of differences in smoothness (Makison and Swan, 2006).

Whitehead and her group in 2005 studied the retention of different bacteria with different cell size included *P. aeruginosa* (1 μm - 3 μm diameter), *S. aureus* (cells 0.5–1 μm diameter) and *C. albicans* (4 μ -5 μm diameter) on coated surfaces with different pits size ranged from 0.2, 0.5, 1 and 2 μm. They found that *S. aureus* was retained in the highest numbers in the 0.5 μm pits size. *P. aeruginosa* on the other hand were mainly retained in the 1 μm surface pits. *C. albicans* were retained in the lowest numbers due to their size which was bigger than the pit size used in the study (Whitehead *et al*., 2005). Recent study by Verran *et al*. (2010) also showed that retention of microbes on the surfaces was dependent on the dimensions of surface features, size and shape of the microbial cell. They tested the effect of feature dimensions on the retention of two bacteria rod-shaped *L. monocytogenes* and coccal-shaped *Staphylococcus sciuri* on titanium-coated smooth surface, and two different width groove surfaces (0.59 μm and 1.02 μm). They found that on smooth surfaces both types of bacteria were present as clusters, and the rod shaped *L. monocytogenes* was

retained in the highest numbers on the 0.59µM surface whereas the coccal-shaped *S. sciuri* cells were retained in the highest numbers to the 1.02 µM linear surfaces. This was due to the available contact area on the surfaces. The possible explanation for the ability of rod-shaped cells to retain in higher numbers on the surface with smaller width features is that the area of contact of the cell with the surface was larger and stronger and if cells are lined up with the surface features, then contact will be available along the length of the cell (Verran *et al*., 2010).

Pathogenic bacteria can be detected from different sources including the air in operating theatres, surgical equipment, health staff clothing, and resident skin microbiota of patients (An and Friedman, 1996). In general, surfaces are considered a non-critical item if they come into contact with healthy skin, as healthy skin is an obstruction to transmission of disease. On the other hand, they may play a role in cross contamination if touched by contaminated hands (Weber and Rutala, 2001). A diagram to show the generic transmission routes of MRSA and VRE is shown in Figure 3 (Otter *et al*., 2011). Numerous studies have demonstrated that surfaces close to infected patients usually become contaminated with MRSA and VRE, and workers may contaminate their hands by touching these surfaces or the opposite could occur (surfaces become contaminated if touched by contaminated hands) (Makison and Swan, 2006, Boyce, 2007, Otter *et al*., 2011). During an outbreak of *A. baumannii* in an intensive care unit in 1998, Denton *et al*. (2004) showed a significant correlation between the number of infected patients with *A. baumannii* and the number of environmental sites, including patient monitors, bed frames, X-ray apparatus, curtain rails, and equipment trolleys" contaminated with *A. baumannii*.

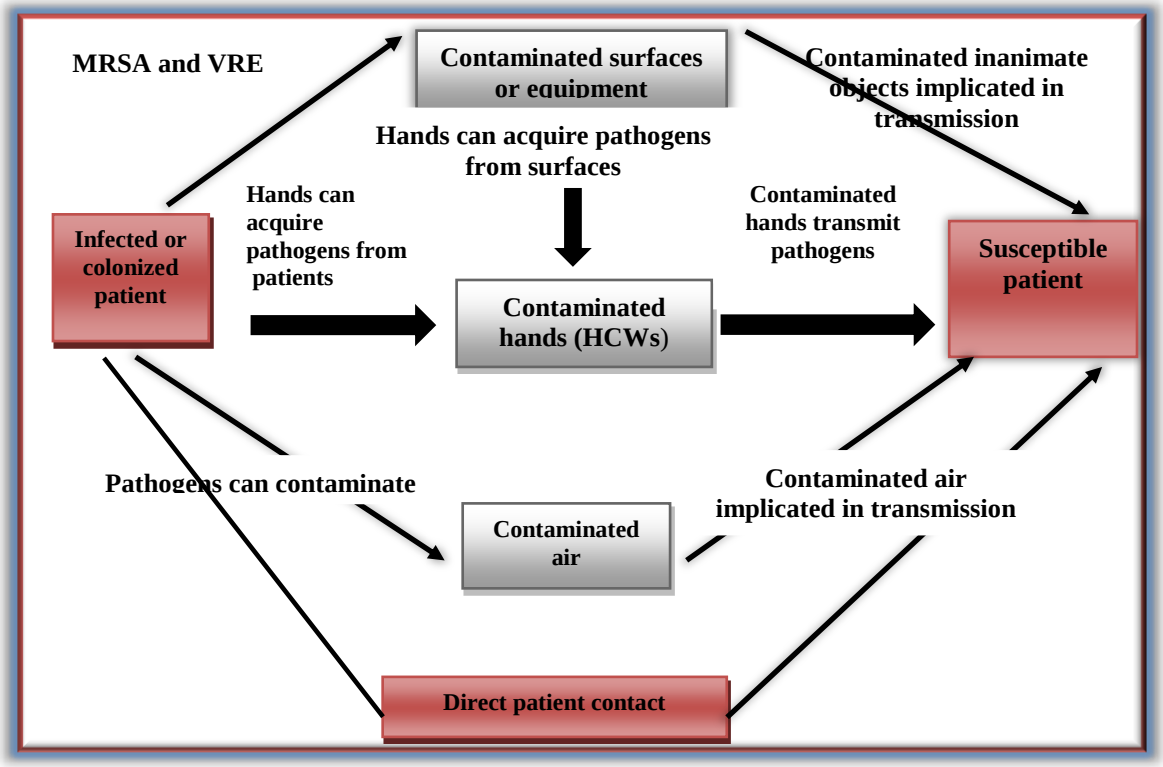

Figure 3 Transmission routes of HCAI. Modified from (Otter *et al*., 2011).

1.5 Surface Classification

Weber and Rutala (2001) defined two different categories of contaminated surfaces based on their role in the transmission of disease; housekeeping and medical equipment. Housekeeping surfaces such as walls and floors are usually associated with the lowest risk of disease transmission. Despite this, there are two different categories of housekeeping surfaces based on the level of contact - high touch surfaces and low touch surfaces. High touch surfaces are associated with the highest risk of transmission of disease (Fuglsang, 2004). Medical equipment such as X-ray machines, blood pressure cuffs and other medical machines form a higher risk in terms of the transmission of diseases than housekeeping surfaces (Weber and Rutala, 2001).

In spite of the lack of direct indications that link HCAIs to environmental contaminants, there is increasing evidence that demonstrates the role of the environment as the main source for some of the pathogens that cause HCAIs. This takes into consideration that touching contaminated surfaces may cause hand acquisition and then the transfer of the contaminant to other surfaces or to patients (Casey *et al*., 2010). Many studies have shown that environmental contamination with MRSA occurs in rooms of either infected or colonized patients (Boyce *et al*., 1997, French *et al*., 2004, Lemmen *et al*., 2004). Moreover, surfaces in patients' rooms such as bed rails and over bed tables are at high risk of contamination if MRSA is present in the palm of the patient's hand (Oie *et al*., 2007).

1.5.1 Concentration of contaminants

The existence of pathogens on a surface does not necessarily represent a transmission risk. However, most environmental infections are associated with low doses of pathogens (Otter *et al*., 2011). Generally, infected or colonized patients have a higher concentration of contamination than their surrounding surfaces (Bonten *et al*., 1996). VRE- positive patients have approximately 10^3 colony-forming units (cfu) 50 cm^{-2} on the skin, while in patients' stools, the concentration ranges from 10^3 to 10^9 cfu- g^{-1} of VRE and MRSA are present (Otter *et al*., 2011). Despite this, touching contaminated surfaces with VRE has almost the same risk for acquisition of VRE on hands as when touching an affected patient. Indeed, unaffected patients who entered VRE contaminated rooms had a high risk of acquisition of VRE (Martinez *et al*., 2003). The infective dose of MRSA varies from study to study. In fact, there is no evidence regarding what is the exact level of surface contamination to be considered as hazardous (Page *et al*., 2009). Consequently, the existence of pathogens on a surface at any concentration may be a chance for transmission (Otter *et al*., 2011).

1.5.2 Survival of nosocomial pathogens on surfaces

Most nosocomial pathogens are capable of survival on surfaces for a longer period than is generally believed, depending on the organism and environmental factors (Kramer *et al*., 2006, Todd *et al*., 2009). These include temperature, humidity, body residues and type of surface material (Kramer *et al*., 2006). For example, the survival time of Enterococci is different on different surfaces. It is able to survive for 4-7 days on countertops, for more than 24 h on the bedrails, 1 h on a telephone headpiece, 30 min on the diaphragm of a stethoscope and more than 1 h on gloves and un-gloved finger tips (Noskin *et al*., 1995). Moreover, Gram-positive bacteria such as *S. aureus* including MRSA, and *Enterococcus* spp. including VRE, are able to survive for months on dry surfaces. On human hands *S. aureus* can survive for at least 150 min and VRE survives for up to 60 min on both hands and gloves. On the other hand, the survival times for these bacteria are longer on inanimate surfaces (7 and 4 months respectively) with wild strains surviving longer than laboratory strains(Kampf and Kramer, 2004). It has been reported that VRE survives for months on plastic surfaces and fabrics (Neely and Maley, 2000). Many Gram-negative species such as *E. coli*, *Acinetobacter spp., Klebsiella spp.,* and *P. aeruginosa* can also survive for months on inanimate surfaces (Kramer *et al*., 2006). *Acinetobacter* is widely known as an outbreak pathogen commonly in intensive care units, and its ability to cause outbreaks is enhanced by its ability to survive for prolonged periods of time (weeks) in different environments (on dry surfaces and in water), and for 60 min on fingertips (Weber *et al*., 2010). In general, Gram-negative bacteria have been characterized as persisting longer than Gram-positive bacteria (Kramer *et al*., 2006),which is due to the different structure of the cell wall which is known to protect bacteria from their surrounding environment. In the Gram-positive bacteria the outer membrane is absent. Therefore, Gram-positive bacteria rarely exist in harsh environments as *E. coli* does (Silhavy *et al*., 2010). Antimicrobial coated surfaces provide health care sectors with a safe environment as they have a natural ability to

tackle the micro-organisms that cause HCAIs and help to reduce their transmission (Figure 4, Page *et al.*, 2009).

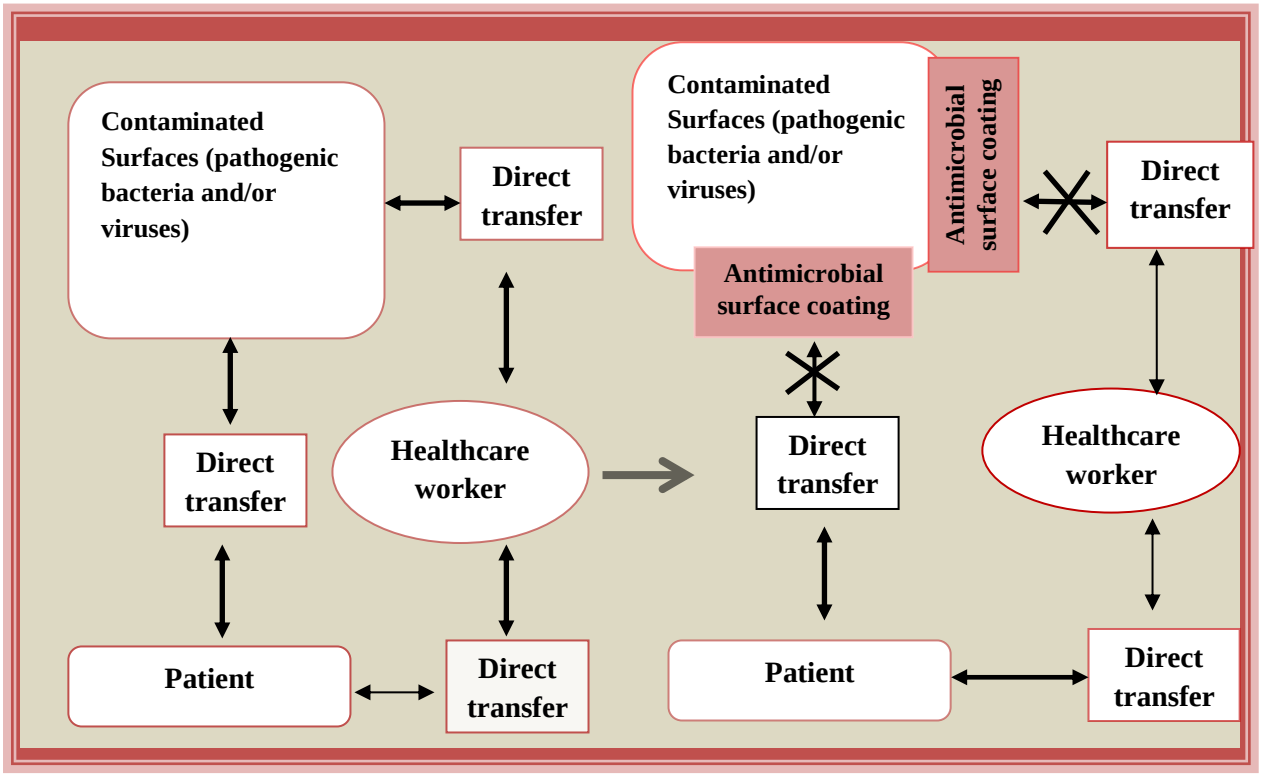

Figure 4 The role of surfaces and antimicrobial surface coating in reducing HCAIs Modified from (Page *et al.*, 2009).

1.5.3 Skin Hygiene

It is known that skin is the largest body organ and works as a protector against invasion by micro-organisms and other toxic materials found in the surrounding environment (Larson *et al.*, 2000). Skin microbiota is a term used to refer to the micro-organisms that colonize the skin. The microbial

distributions and sum of skin microbiota vary, depending on sex, age, health condition and site on the body (Katz, 2004). Bacteria are associated with human body fluids so the survival of bacteria would increase if albumin, sugar, and serum where present (Jawad *et al*., 1996). Indeed, dark and high humidity also lead to longer survival (Kramer *et al*., 2006).

1.5.4 Human hand

Hand microbiota are divided into two categories; resident and transient microbiota(Kampf and Kramer, 2004). Resident microbiota is associated with micro-organisms that are able to survive and reproduce on both superficial and deep skin layers. Therefore, it is not easy to eliminate them by hand washing. However, some penetrating disinfectants may be able to eliminate micro-organisms by killing them or inhibiting their growth (Filetoth, 2003). Resident microbiota on human hands includes numerous bacterial species, including Gram-positive micrococcaeae and Gram-negative species, such as *Acinetobacter* and *Klebsiella*. Despite this, resident microbiota are considered safe in terms of human health due to innate skin resistance. However, they may cause clinical infection if skin immunity is lowered (Pittet, 2001, Filetoth, 2003).

Transient microbiota are deposited on the skin but do not colonise (Lilly and Lowbury, 1978), and they typically consists of Gram-negative species and are detected in external skin layers only. They mainly colonise hands or skin sites that come into contact with an external source which may include infectious agents (Filetoth, 2003). Most are more sensitive to hand washing than resident microbiota but have higher infection potential (Lilly and Lowbury, 1978, Vesley *et al*., 1985). In fact, resident microbiotas also survives on superficial skin (and in deep skin) and are as sensitive as the transient microbiota to removal by hand washing. Therefore the definition used to differentiation between them is not idealistic (Vesley *et al*., 1985).

The transmissibility of transient bacteria is controlled by the bacterial species, the total count of bacteria on hands, the survival period on the skin and the degree of wetness (Merry *et al*., 2001). A study by Patrick *et al*. (1997) showed that the number of bacteria transferred between surfaces was reduced by 99% when dry hand procedure was followed. Moreover, ineffective hand drying could lead to skin excoriation which could cause an increase and a change in bacteria that colonise the skin (Snelling *et al*., 2011). A diagram to show a common way of transmission from inanimate surfaces to susceptible patients is shown in Figure 5 (Kramer *et al*., 2006).

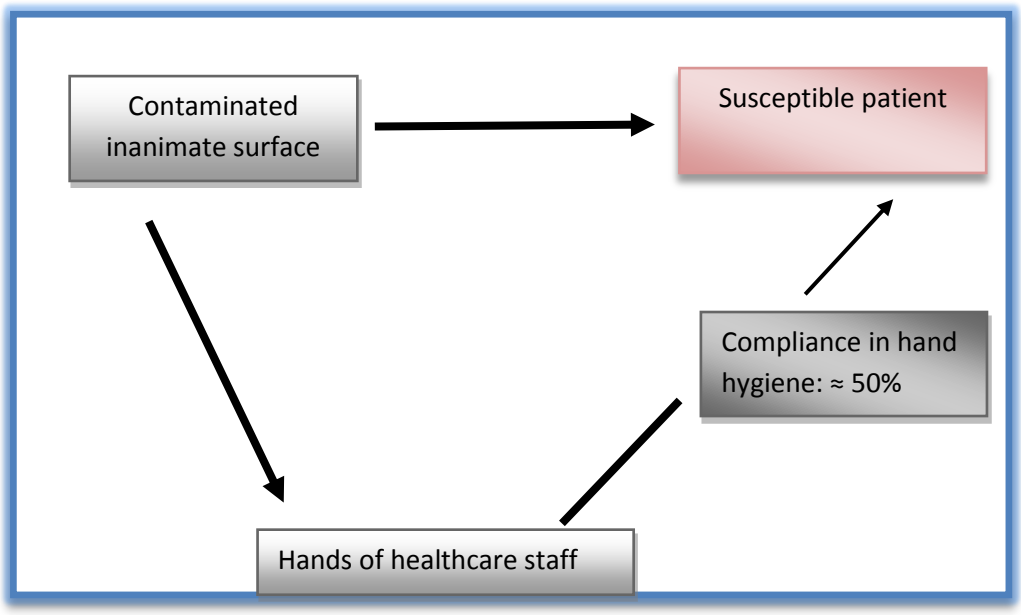

Figure 5 Common ways of transmission from inanimate surfaces to susceptible patient

Modified from (Kramer *et al*., 2006).

A study by Fierer *et al*. (2010) demonstrated that a typical hand carries about 150 bacterial species. These mostly colonise under finger nails and in the palm of the hand. The under finger nails area is an occluded area and has a sufficient amounts of moisture to encourage the growth of bacteria and

fungi (McGinley *et al*., 1988). Moreover, artificial nails and chipped nail polish may lead to an increase in the number of bacteria on the finger nail area (Wynd *et al*., 1994).

The human hand has long been identified as an important route in the transmission of pathogenic organisms associated with hospital infections, even between patients, health workers and the public (Larson *et al*., 2000, Katz, 2004) , which is due to the ability of these organisms to persist on hands for prolonged periods .The most common pathogenic organism that spreads via healthcare workers are *S. aureus* and Gram-negative bacilli (Weinstein and Hota, 2004). It has been reported that the contamination of hands of health staff was higher after contact with patients colonized with Gram-positive compared to the Gram-negative bacteria. This may be due to the fact that the environmental spread of Gram-positive bacteria is more extensive due to their viability; the majority of normal skin microbiota consisted of Gram-positive bacteria (Lemmen *et al*., 2004). This despite the fact, that a number of guidelines for the determent of the spread of multi-resistant pathogens in the hospital are currently used. However, none of them differentiate between Gram-positive and Gram-negative (Lemmen *et al*., 2004). The transfer rates of micro-organisms from nonporous surfaces were lower than from porous surfaces. Since porous surfaces such as a sponge, offer many deep recesses in which micro-organisms can hide and become less accessible to the human hand. In contrast, a nonporous hard surface (smooth surface) does not offer cracks in which micro-organisms may hide; thus their transmission is low compared to those on a porous surface. In spite of their low rate of transmission ability, porous surfaces caused high level of hand contamination. After squeezing out a sponge as an example of porous surfaces, the subject's hands were highly contaminated. Therefore, evaluating the transfer efficiency rates of different types of pathogenic organisms from surfaces to surfaces is the first step to assess the risk of transmission from contaminated surfaces (Rusin *et al*., 2002).

1.6 Antimicrobial surfaces

An antimicrobial surface is a surface that has a natural antimicrobial activity that affects the ability of microorganisms to survive or proliferate and which reduces the risk of transmitting disease, poisoning the environment, or biofouling materials (Ho *et al*., 2004). Such materials could be used to coat the surfaces of objects that are used and touched regularly by people in everyday life such as door knobs, computer keyboards, telephones, and children's toys to make them antiseptic and unable to transmit bacterial infections (Tiller *et al*., 2001), hence, they have been studied widely. They are used frequently in the health, clothing, food and public transport industries (Nepal *et al*., 2008).

1.6.1 Antimicrobial coated surfaces

Antimicrobial coated surfaces are another way of reducing microbial contamination of the environment (Casey *et al*., 2010). There are many different techniques used for coating surfaces, such as sol-gel, magnetron sputtering and chemical vapour deposition (CVD) (Foster *et al*., 2010). CVD has been widely used for many years across an extensive range of industrial applications to produce thin film coatings. In such a process a reactive gas mixture is introduced in the coating region, and a source of energy applied to initiate (or accelerate) a chemical reaction (usually thermal or plasma), resulting in the growth of a coating on the target substrate (Choy, 2003). CVD has many advantages compared with other techniques; it uses large scale equipment, gives a high quality coating (hard, durable and highly active) and uses inexpensive equipment (Gordon, 1997, Yates *et al*., 2008). In this study, copper deposited with silica was made by using the flame assisted chemical vapour deposition (FACVD) process which does not require closed reaction cells (Yates *et al*., 2008).

Flame assisted chemical vapour deposition (FACVD) is a subtype of CVD. This method is based on the use of a flame and combustion process (precursor liquid or gas delivered into flames) as a source

of thermal environment required for vaporization, decomposition, and chemical reaction. The flame temperature is usually very high, ranging between 1700–2730° C. The flame temperature and its distribution, which usually causes the homogeneous gas phase reaction to occur leading to the deposition of powders, are the main process elements that can control the crystal structure, morphology, and particle size. Hence, the FACVD is widely used commercially for the production of powder. The FACVD can be differentiated from the conventional CVD in that the time required for the process of coating films (vaporisation, decomposition, and chemical reactions) is shorter due to the flame, which helps to heat the substrate to enhance diffusion on the surfaces of the substrate during the deposition of the films. The main drawback of the FACVD method is the large temperature fluctuation of the flame source during deposition due to the large temperature gradient present in the flame. However, modified methods have been produced by developing specially designed burners to produce a flat and uniform flame such as the counter flow flame burner. FACVD has long been used by the oxide powder industry to produce large quantities of powders of TiO_2 and SiO_2 using metal chloride precursors in hydrocarbon flames due to the viability and low production cost (Choy, 2003).

The variant of Atmospheric Pressure CVD (APCVD) has established itself increasingly in recent years, as a technologically and commercially attractive sub-set of CVD coating. It has been particularly successfully employed in production of coating processes in a wide range of industrial applications such an on-line glass coating, tool coating, ion barrier layer deposition, anti-corrosion and adhesion layers on metals, anti-scratch coatings on bottles etc. On-line CVD films are known for their hardness, which is a major advantage in subsequent industrial processing and in many of the target applications(Gordon, 1997).

1.6.2 Classification of surface coatings

Based on their functionalized use against microorganisms, antimicrobial surfaces can be classified into two different categories ; anti-adhesive and biocide releasing surfaces (Ho *et al*., 2004) . The anti-adhesive coated surfaces act as a preventer that blocks microbial adhesion to the surfaces, such as coating the surfaces with a layer of polyethylene glycol (PEG) (Page *et al*., 2009). Even though these surfaces have strongly reduced microbial adhesion, they never show 100 percent reduction (Ho *et al*., 2004). Materials containing triclosan, silver, and copper are most commonly used to kill microorganisms based on the release of a biocide. Triclosan, which is found mainly in personal care products as well as touchable surfaces like chopping boards, works more as a disinfectant (killing outside in) rather than an antibiotic (killing inside out) which means that its activity is not permanent (Page *et al*., 2009). Silver is able to express its antimicrobial activity by release of ions or as a contact active material (Ho *et al*., 2004). However, the unnecessary release of biocides may cause increases in both environmental contamination and microbial resistance. Therefore, many different types of modern contact active material have been modified to kill microbes on contact without releasing a biocide. Modification can be achieved by grafting the surfaces using chemical antimicrobial polymers such as N-alkylated poly (4-vinylpyridine), and quarternized poly(ethylene imine), and acrylates (Fuchs and Tiller, 2006, Waschinski *et al*., 2008) to various typical materials such as glass, cellulose and plastics (Fuchs and Tiller, 2006). The principle of the modification method is based on a polymer with one biocidal and one polymerizable end group (Waschinski *et al*., 2008). Moreover, light activated antimicrobial surfaces such as a titanium dioxide based photocatalytic coatings can be interpreted as contact-killing surfaces that kill microbes by light-induced production of reactive oxygen species (ROS) (Ho *et al*., 2004). ROS have no specific target in terms of microbes. Therefore, this avoids the potential problems of microbes developing resistance

to microbiciodal treatment and has the advantage of also having self-cleaning properties (Page *et al*., 2009).

1.6.3 Silver surfaces

Silver is among the inorganic antibacterial agents that has been known and used to fight infection for centuries. Silver coins and vessels were used by the Grees and Romans to maintain water purity. Silver nitrate solution (1%) was commonly applied to new-borns' eyes to prevent infections that lead to blindness (Page *et al*., 2009). Due to its antimicrobial and anticaries properties, silver has been widely used in a variety of medical applications included dentistry fillings (Peng *et al*., 2012). It is also used to coat some medical devices that are implanted inside the human body such as the coating of catheters (Silver *et al*., 2006).

The use of silver in wound treatment dates back to the 18th Century when silver nitrate was used to treat sores. Silver was accepted by the US Food and Drug Administration as an effective agent for wound treatment in the 1920s. However, the used of silver fell after the introduction of antibiotics (penicillin) in the 1940s. Silver (silver nitrate) was once more used for the management of burns in the 1960s in combination with sulphonamide antibiotic to produce silver sulfadiazine cream which has a broad spectrum antimicrobial ability (Chopra, 2007). Recently, silver has been widely used for clinical purposes due to the development of microbial resistance to antibiotics (Chopra, 2007). Silver nitrate has also been used for reducing and preventing caries in primary and permanent teeth. The use of silver in combination with fluoride was also reported as an anti-caries agent. However, the use of silver fluoride compounds has been limited to clinical application due to the black staining associated with caries lesions (Peng *et al*., 2012).

Silver is considered to be an antimicrobial agent due to its efficacy against a range of micro-organisms and the lack of toxicity to non-targeted cells. Silver ions have been introduced into a wide

range of materials, which leads to an extensive and growing range of silver-based antimicrobial products (Taylor *et al*., 2009). It has also been used for a number of non-medical purposes such as in electrical appliances, the linings of washing machines, dishwashers and toilet seats (Jung *et al*., 2008). It can be used in various moulded plastic products, in textiles and in coating-based applications, including countertops and food storage and preparation areas (Egger *et al*., 2009).

It has been reported that silver-treated materials can reduce levels of bacterial contamination in healthcare settings. There has been a 96% reduction in the bacterial count on silver treated material compared with a 44% reduction on untreated surfaces (Taylor *et al*., 2009).

Even though silver demonstrates a broad spectrum antimicrobial activity, the increased use of silver in medical settings has raised concern about the potential widespread of microbial silver resistance. It has been reported that certain types of microorganisms have developed a resistance to silver. These included *E. coli*, *Enterobacter cloacae, K. pneumoniae, A. baumannii*, and *S. typhimurium* (Percival *et al*., 2005). In a recent study, catheters coated with silver demonstrated a significant antimicrobial activity reduction against all microorganisms tested (*E. coli, S. aureus, P. aeruginosa*, coagulase-negative staphylococci and *Enterococcus*) for 72 h. They showed complete inhibition of cell growth for almost all the microorganisms tested, with the exception of *P. aeruginosa* where cell growth was reduced by 67%. Biofilms of coagulase-negative staphylococci (which are the most common cause of catheter related infections), *Enterococcus*, and *P. aeruginosa* were reduced by more than 50% compared with almost 100% reduction for other organisms (Roe *et al*., 2008).

Woods *et al.* (2009) showed that of 176 *Enterobacter cloacae* strains tested, only 6 strains were silver resistant. The fact that silver-resistant genes are encoded by the same plasmid that encodes traditional antibiotic resistance, leads scientists to be concerned about increasing silver resistance due to the potential of cross resistance with other bacteria (Woods *et al*., 2009). However, despite the

widespread use of silver, there has not been a large increase in Ag resistance in pathogens which may be due to the multiple sites of action of Ag.

1.6.3.1 Silver toxicity

The antimicrobial activity of silver is dependent on the silver cation Ag^+. Therefore, silver-based antimicrobial polymers have to release silver ions to the environment in order to be effective (Kumar and Münstedt, 2005). Ag^+ causes the release of K^+ from bacteria. Therefore, the bacterial plasma or cytoplasmic membrane is an important target site of Ag^+. When Ag^+ enters the bacterial cell, they inhibit cell division and damage the cell envelope. And when exposed to effective concentration of silver, the size of the bacterial cells increases, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers display structural abnormalities (Kim *et al*., 2011). Ag^+ has a strong ability to bind to electron donor groups in biological molecules such as amino, phosphate, carboxyl or thiol groups in proteins or in DNA (Gordon *et al*., 2010). It has been shown that the interaction of silver with thiol groups play an essential role in bacterial inactivation. A recent study has evaluated the relevance of the potential targets for the bactericidal effect of silver. They found that additional amounts of potassium phosphate and excess DNA did not reduce the antimicrobial activity of the silver, whereas additional amounts of the thiol group containing the amino acid cysteine, but not other amino acids such as glutamate, which lacks sulphur, terminate the bactericidal activity of silver. In the same study, the researchers also showed that respiratory chain enzymes which bound to the cell membrane are inactivated by silver, mainly iron-sulfur clusters (Gordon *et al*., 2010). Bragg and Rainnie (1974) showed that silver ions cause damage to the respiration chain and demonstrated that there were two sites in the respiration chain that were sensitive to silver ions. The site located between the *b*-cytochromes and cytochrome a_2 was more sensitive than that found between NAD^+ or succinate and flavoprotein, which may due to the fact that several sulfhydryl groups with different re-activities were present in the dehydrogenase regions of the respiration chain (Bragg and Rainnie,

1974). Dibrov *et al*. (2002) reported that bacterial death was due to proton leakage through the bacterial membrane as an effect of low concentration of silver. They demonstrated that the bactericidal effect of such a concentration was not mediated by a specific target in the cell, but was due to proton (H^+) leakage which occurs through any Ag -modified membrane protein or perhaps through the Ag-modified phospholipid bilayer itself. Since the trans-membrane proton gradient controls the overall microbial metabolism, there is no doubt that the proton leakage effect of Ag would result in cell death. Inoue *et al*. (2002) investigated the bactericidal activity of Ag-zeolite against *E. coli* under aerobic conditions and showed that dissolved oxygen was necessary for the antimicrobial action of silver and that reactive oxygen species (ROS) played an important role in the bactericidal activity. They showed that dissolved oxygen might be reduced to form superoxide anions, hydrogen peroxide and hydroxyl radicals. This study looked at the effect of adding two different ROS scavengers, catalase and sodium benzoate (hydrogen peroxide and hydroxyl radicals respectively), on the bactericidal effect of silver. The results showed that there was no cell count of *E. coli* when no scavenger was added. However, in the presence of catalase, the viable cell count of *E. coli* in the suspension was similar to that in the controls condition, thus the addition of catalase stopped the bactericidal activity of silver completely. A recent study by Jung *et al*. (2008) found that Ag^+ killed both *E. coli* and *S. aureus*, but over a different period. *E. coli* was more sensitive and was killed within one hour, whereas three hours were required for *S. aureus* to be killed. This was due to the thick peptidoglycan layer within the cell wall of *S. aureus*. Kawahara *et al*. (2000) investigated the effect of silver on oral bacteria under anaerobic conditions. They showed that gram-negative species (*Porphyromonas gingivalis*, and *Prevotella intermedia*- periodontal-pathogens) were more sensitive to the silver than Gram-positive bacteria (*Streptococcus mutans, Streptococcus sanguis, Actinomyces viscosus*- cause dental caries). The resistance of Gram-positive species was due to the

thickness of peptidoglycan layer which are negatively charged and allow fewer silver ions to pass through the plasma membrane than Gram-negative species.

1.6.4 Copper as an antimicrobial agent

Copper is a reddish metal that is found naturally in rock, soil, water, and sediment (Dorsey *et al*., 2004). The average concentration in the earth's crust is about 50 parts copper per million parts soil, and it presents in nature in four oxidation states; solid metal Cu (0), Cu (I) cuprous ion, Cu (II) cupric ion, and rarely Cu (III) (Kiaune and Singhasemanon, 2011). It is also found naturally in all plants and animals. Metallic copper is easy to mould or shape. Copper can also be found in many mixtures of metals, termed alloys, such as brass and bronze. Copper compounds are also found, and these include both naturally occurring minerals and manufactured chemicals. Indeed, copper sulphate is the most commonly used copper compound (Dorsey *et al*., 2004).

Copper has been known as an antimicrobial agent since ancient times, well before microorganisms were discovered in the 19th century, and produced successful results when used by doctors in surgical wounds in the early 1800s. Moreover, the first time copper was used in medicine as a biocide was by an Egyptian doctor recorded in the Smith Papyrus around 2600 and 2200 BC (Grass *et al*., 2011). The Phoenicians used copper and silver bottles to store wine, water, and vinegar and in World War I, copper was used to prevent wound infection (Gabbay *et al*., 2006). Today, copper and copper alloys are widely used as chemical biocides for medical and non-medical purposes. They are used as bactericides to act as self-disinfectants in paints, to purify water distribution systems with regard to *Legionella* in hospitals (Borkow and Gabbay, 2004), as a fungicide in agriculture to protect some plants such as coffee, tea, citrus and cocoa from fungal leaf diseases (Cervantes and Gutierrez-Corona, 1994, Kiaune and Singhasemanon, 2011), and as an active ingredient in many pesticide

formulations mainly after the tributyltin was banned in the late 1980s (Kiaune and Singhasemanon, 2011). These days, copper alloys are also widely used on coating surfaces, not only because they exhibit a strong antimicrobial activity against different microbes but also due to many other characteristics, including their availability in a range of different colours, and because they are easily alloyed, and highly recyclable (Michels *et al*., 2005).

Copper surfaces have been shown to kill a variety of pathogens including *S. enterica* and (Faundez *et al*., 2004), *Listeria* (Wilks *et al*., 2006), MRSA (Gould *et al*., 2009, Michels *et al*., 2009, Noyce *et al*., 2006, Wang *et al*., 2004), *E. coli* O157 (Gogniat and Dukan, 2007, Guan *et al*., 2003), *K. pneumoniae* (Mehtar *et al*., 2008) and enterococci (Gould *et al*., 2009, Warnes and Keevil, 2011).

Re-colonisation of copper surfaces following cleaning was delayed compared to control surfaces (Tiller *et al*., 2001). However, copper surfaces may become conditioned allowing colonisation following cleaning, possibly because of the reaction of copper with cleaning products. Therefore, if copper is to be used in the hospital environment as an antibacterial surface, the type of cleaning and disinfection should be considered (Airey and Verran, 2007). The killing of microbes on copper surfaces is known as "contact killing", and the high antimicrobial efficacy of copper-coated surfaces could be due to some of its natural features such as wear resistance, being solid and homogenous, easy to clean and durable (Grass *et al*., 2011).

In addition to its use on a contact surface, the antimicrobial effect of copper is being introduced to a number of other applications, such as in the form of the introduction of copper oxide into textiles (sheets and clothing) and latex (O'Gorman and Humphreys, 2012). Face masks are commonly used in healthcare settings, either by health works and/or doctors, or by patients, to protect themselves from respiratory infection. These masks are also frequently worn in crowded areas for such purposes as to protect the wearer from environmental contamination or to protect the environment from the

wearer's infection. Most of them contain a non-woven layer that, based on its pore size, prevents the passage of pathogens through the mask. It has been demonstrated that the addition of copper oxide into face masks reduces the risk of influenza virus environmental contamination without changing the filtration capacities of the masks. These types of masks are considered safe to use, not only due to their antiviral and antimicrobial activity, but also due to the fact that the amount of copper eluted to the air from the masks were very small (0.47pg) and below the respiratory copper permissible exposure limit ($>10^5$ fold) set by the USA occupational Safety and Health Administration (Borkow *et al*., 2010).

In another clinical study, copper-impregnated socks were been tested against fungal foot infections (*Tinea pedis*). The researchers found that within 1-3 days of wearing copper impregnated socks (10% copper), all one hundred patients' feet had recovered, and the burning and itching which may accompany the fungal infection had disappeared. In the case of acute and chronic infections, a longer period of time was required, ranging from 2 to 6 days (acute infection) and 1 to 2 months (chronic infection) depending on the cause (Gabbay *et al*., 2005). Borkow and Gabbay (2004) reported 2 log viable cell reductions of *E. coli* and *S. aureus* within 2 h of exposure to copper fabrics. They also showed that extensive washing (35 industrial washings at 85°C in a tumble cycle using abrasive salts and soaps) of copper fabrics had no effect on their antimicrobial activity, and still gave 2 log reductions for both types of bacteria within 2 h. In the same study, the copper-impregnated fabrics did not cause skin irritation on animal skin that was exposed to copper fabric for 4 h (Borkow and Gabbay, 2004). Others reported the efficiency of copper biocide/Aloe Vera-based biocidal hand rubs (Xgel) against the bacteria responsible for HCAIs, including MRSA, and *A. baumannii*, compared to the hand rubs commonly used in hospitals. They showed that copper-based hand rub was more active as a biocide and caused less irritation to hands (Hall *et al*., 2009).

In addition to laboratory testing which has proved the ability of copper to continuously kill bacteria that cause infections and indicated it to be the most effective touch surface, clinical trials are now under way around the world demonstrating the benefit of antimicrobial copper in real life use conditions. These trials are taking place in many hospitals settings around the word including the US, the UK, Chile, Germany, South Africa and Finland. Results from the above trials show that microbial contamination is significantly and consistently reduced by 83-100% on copper compared to standard surfaces (Anon, 2012).

In the US trial, three different medical centres were involved in assessing copper's antimicrobial efficacy in intensive care units (ICUs) - the Medical University of South Carolina, Charleston (MUSC), The Ralph H. Johnson Veterans' Administration Medical Centre, Charleston, South Carolina and the Memorial Sloan Kettering Cancer Center in New York City. In these trials, common touch surfaces which are usually made from stainless steel, aluminium and plastic were replaced with antimicrobial copper alloys. These included bed rails, over bed tray tables, chairs, call buttons, data devices and IV poles in selected rooms of the ICU departments (16 rooms). The trial was conducted over a period of 43 months and no changes were made to cleaning regimes in the study rooms. Surfaces were sampled over the period of testing. The results showed that the introduction of the copper alloys led to an 83% reduction in the average microbial burden compared to the controls (Schmidt *et al*.,2012b).

In the UK, a clinical trial took place at Selly Oak Hospital, Birmingham, and lasted for 10 weeks. In this trial, commonly-touched surfaces such as toilet seats, tap handles and door push plates were replaced with 60% copper surfaces. Surfaces were sampled on a weekly basis for the presence of microorganisms. The first results showed that surfaces made from materials containing copper had 90 - 100% fewer microorganisms compared with the same items made from standard materials

(chrome-plated brass, aluminium and plastic-control surfaces). Results also showed that pathogenic bacteria such as meticillin-susceptible *Staphylococcus aureus* (MSSA), VRE and *E. coli* were detected only on the control surfaces (Casey *et al*., 2010). Similar results were also reported from a second extended phase of this hospital trial which was carried out over 24 weeks. In this trial, fourteen types of frequently touched items made of copper alloy were installed in various locations on an acute care medical ward, and were sampled once weekly for 24 weeks. After 12 weeks of sampling, copper and standard items were switched over to reduce bias in usage patterns. The study showed that the microbial count on all copper items was lower compared with counts on standard materials. However, 8 of them were significantly reduced, whereas on the other 6 copper items, reduction did not reach statistical significance. The study also showed that the indicator organisms MRSA, methicillin-susceptible *Staphylococcus aureus* (MSSA), VRE, and coliforms were found on both surfaces; however, significantly fewer copper surfaces were contaminated with VRE, MSSA, and coliforms, compared with control surfaces (Karpanen *et al*., 2012).

In Chile, a trial was held in intensive care unit at the Hospital del Cobre, in Calama for 30 weeks. In this trial, bed rails, bed levers, tray tables, chair arms, touch screen monitor pens, and IV poles were replaced with copper and placed in selected ICU rooms. The results of this trial showed that copper was effective in reducing microbial loads on all the surfaces tested (bed rails by 91%, bed levers by 82%, tray tables by 83%, chair arms by 92%, monitor pens by 49% and IV poles by 88%). In copperized rooms, the microbial surface count was significantly lower than in rooms without copper. Copper was effective in reducing the staphylococcal burden which was the most predominant microorganism isolated. MRSA and VRE were not isolated on copper surfaces (Prado *et al*., 2010).

In Finland, a trial was conducted at a nursing home in conjunction with the Helsinki University Department of Public Health. The contamination of copper items including dressing trolleys, door

handles, grab rails, handrails; shower drains and push buttons was compared with standard items in patients' rooms, bathrooms and communal areas. The results showed the levels of contamination on the non-copper items were higher compared with copper surfaces, and faecal and urinary bacteria, such as *S. aureus*, and *E. coli were* present only on non-copper surfaces (stainless steel, plastic and chromium). On copper surfaces, only Gram-positive bacilli and cocci and normal environmental and skin microbiota were present (Anon, 2012).

In Germany, a 32 week trial was carried out in the Asklepios Clinic, in Hamburg. Touch surfaces included aluminium door handles and plastic light switches which were replaced with copper alloys (percentage of copper alloy not stated). The study found that the total number of cfu on metallic copper surfaces was reduced by 63% compared with the control surfaces (Mikolay *et al*., 2010).

In South Africa, a six month trial was conducted in the consulting rooms of a walk-in primary care clinic in Grabouw. High and less frequently-touched surfaces such as a desk, trolleys, the top of a cupboard and windowsills were covered with copper alloy sheets (99.9% copper). The surfaces were sampled every six weeks with multiple samplings per day. The results showed an overall 71% reduction in bacterial load on the copper surfaces compared the control surfaces during the working day, and only over weekend periods (71 h) when the clinic was closed and microbial loading was markedly reduced, the survival of microorganisms on both surfaces was comparable (Marais *et al*., 2010).

1.6.4.1 Role of copper in reducing HCAIs rate

Cleaning is an effective way to reduce the bacterial burden (BB) on surfaces and minimize the infection risk to patients. However, BB can rapidly return. Copper, when used on hospital surfaces (such as bed rails), was found to continually reduce surface BB before and after cleaning due to its continuous antimicrobial activity (Schmidt *et al*., 2013).

It is well documented that patients in ICU are at high risk of HCAI because of severity of illness, and frequent interaction with healthcare workers (HCWs). In addition, patients in rooms with high bio burden were more likely to develop HCAI than those in rooms with low bio burden. This may due to that the persons with active infection are more likely to shed bacteria captured by environmental. A recent study showed that placing a copper alloy surface onto 6 common, highly touched objects in ICU rooms reduced the risk of HCAI by more than half at all study sites (Salgado *et al*., 2013). The percentages of HCAI and/or colonization with MRSA or VRE on patient admitted to copper rooms were lower than that among patients admitted to non-copper rooms. Both MRSA and VRE colonization were decreased by 2.7-fold among patients admitted to copperised rooms. The authors believe that HCAI reduction was due to the continuous antimicrobial effect of copper on environmental pathogens together with standard infection prevention practices commonly used in hospital (Salgado *et al*., 2013). Researchers in another study demonstrated that the use of textiles impregnated with copper oxide in a long-term care ward may significantly reduce the rate of HCAI, fever, antibiotic consumption, and related treatment costs. They compared the rates of HCAI in two patient groups in a head injury care ward before and after replacing all the regular linens and personnel uniforms with copper oxide impregnated biocidal products. They found that per 1000 hospitalization-days there was a 24% reduction in the HCAI, a 47% reduction in the number of fever days (>38.5°C), and a 32.8% reduction in total number of days of antibiotic administration. In addition, there was approximately 27% saving in costs of antibiotics, HCAI-related treatments, X-rays, disposables, and laundry (Lazary *et al*., 2014). Therefore the introduction of copper surfaces to objects found in the patient care environment will provide a potentially safer environment for hospital patients, HCWs, and visitors (Schmidt *et al*., 2012b).

1.6.5 Copper toxicity

Copper is one of the metallic elements essential for human health. The adult body contains between 1.2 and 1.4 mg of copper per kg of body weight and it is estimated that a human eats and drinks about one mg of copper every day with excess amounts of copper released in bile and excreted in faeces (Borkow and Gabbay, 2005). At low concentrations, copper is an essential element for all living organisms due to its role in many reactions. It acts as a co-factor for many proteins (Faúndez *et al*., 2004, Grass *et al*., 2011), in electron transport as an electron donor or acceptor and as an electron carrier, and in oxidation reactions. It is a cofactor for over 30 known enzymes in higher organisms due to its ability to cycle between Cu^{+2} and Cu^{+1}. Some examples are lysyl oxidase, which involved in the cross-linking of collagen, tyrosinase, required for melanin synthesis, cytochrome c oxidase act as a terminal electron acceptor of the respiratory chain, and superoxide dismutase which is required for defence against oxidative damage. Other copper proteins act as electron carriers, such as plastocyanins and azurins (Solioz *et al*., 2010). The ability of copper to convert from cuprous Cu (I) (reduced) to cupric Cu (II) (oxidized) during oxidation-reduction is controlled by environmental chemical compounds (Cervantes and Gutierrez-Corona, 1994). For example, an increased pH level in the environment accelerates copper toxicity, due to the reduced competition between copper and hydrogen ions at the cell surfaces. Whereas some cautions such as Ca^{+2} and Mg^{+2} present in the environment cause a reduction in copper toxicity, because they compete with Cu^{+2} for biological binding sites (Kiaune and Singhasemanon, 2011). On the other hand, at high concentrations, copper is toxic to living organisms including human. In the human body, copper can cause liver and kidney damage and even death in some cases. Drinking water containing large amounts of copper may cause vomiting, stomach cramps, or diarrhoea. In addition, long time

exposure to copper dust can cause nose, mouth and eye irritation, and cause headaches, dizziness, nausea and diarrhoea (Anon, 2004).

Microorganisms have developed complex systems including specific uptake and efflux pumps which represent the largest category of metal resistance systems. Microorganisms use active transport mechanisms to export toxic metals from their cytoplasm and they can be non-ATPase or ATPase-linked in order to maintain precise intracellular levels of copper. Many other mechanisms in addition to specific uptake and efflux pumps are also used. These include exclusion through the use of a permeability barrier (changes in the cell wall, membrane or envelope of the microorganism), intra- and extracellular sequestration by protein binding, and enzymatic detoxification to less toxic forms. The genes responsible for these processes may be encoded by the chromosome or by plasmids, and microorganisms can use one or a combination of several resistance mechanisms (Borkow and Gabbay, 2005). Researchers have reported the presence of *tcrB* gene (a copper resistance gene) in certain strains of *E. faecium* and *E. faecalis*. They showed that these strains are able to grow on agar plates containing high concentrations of copper sulphate. However, their resistance is weak, and does not prevent them from dying when exposed to copper surfaces (Hasman and Aarestrup, 2002). Santo *et al*. (2008) demonstrated a reduced death rate with regard to *E. coli* strains containing copper resistance plasmid (*PCo*) when exposed to copper surfaces. Even though such a copper resistant gene can be encoded by transmissible plasmids, the potential for widespread bacterial strains resistant to copper surfaces appears unlikely due to the rapid rate of contact killing (Santo *et al*., 2008).

The toxicity of copper is mainly due to its natural properties (the redox properties with inherent toxicity). As previously stated, redox cycling between copper ions (Cu^{+2} and Cu^{+1}) can catalyse the production of highly reactive hydroxyl radicals, which can subsequently damage lipids, proteins,

DNA and other biomolecules (Borkow and Gabbay, 2005). The precise mechanism by which copper exerts its biocidal activity is still not clear but is thought to be multi-factorial rather than the result of a single universal mechanism (O'Gorman and Humphreys, 2012). These factors include the combination with proteins that do not require copper which leads to the breakdown of the osmotic balance and the disturbance of membrane components (Cervantes and Gutierrez-Corona, 1994, Santo *et al*., 2011) and binding to specific sites in nucleic acids (DNA) which causes strand breaks and base modification (Yates *et al*., 2008). Recently, researchers have proved that dry copper and copper alloy surfaces demonstrate the highest percentage of killing of a wide range of pathogenic microbes compared with other surfaces (Casey *et al*., 2010, Grass *et al*., 2011, Santo *et al*., 2011). It has also been proved that pure copper kills 100 % of MRSA, *E. coli* and *L. monocytogenes* within 45 min (Wilks *et al*., 2005). It has also been reported that copper is toxic to ESBL-producing organisms. The CTX-M-15 (beta-lactamase active on cefotaxime) producing *E. coli* and NDM-1(New Delhi Metallo beta-lactamase) producing *K. pneumoniae* were killed on copper surfaces within 60 min of incubation at room temperature (five-log reduction). However, 60 min killing time is much less efficient than one min to kill a wild type strain of *E. coli* at room temperature as shown previously by Santo *et al*. (2008). The different results may be due to different assay protocols and use of alloys with different copper contents. In Santo's report, instead of using liquid solution, cells were spread across copper coupons using cotton swabs and allowed to dry.

Copper ions are considered by many researchers as the main cause of cell destruction (Grass *et al*., 2011, Santo *et al*., 2011). Moreover, authors in a recent study have proposed that cell proliferation and cell differentiation are Cu ion concentration dependent. However, the excess of Cu ions may cause cell death and reduce the differentiation of osteoblastic cells (in cases where copper is used for implant application; (Liu *et al*., 2014). Therefore, the balance in the concentration of Cu ions between antimicrobial activity and cytotoxicity should be considered as an important factor for the

development of antimicrobial surfaces (Liu *et al*., 2014, Wu *et al*., 2014). Copper can penetrate into bacterial cells through their envelope as shown in Figure 6. Santo *et al*. (2011) showed that the levels of copper ions taken up by *E. coli* cells remains high throughout the killing process, suggesting that the cell destruction was due to the overload of intracellular copper. Further research is necessary to define the correlation between copper content and bacterial killing more accurately and to identify the best alloys for use in health settings. Copper could be used as a self-sterilizing material on surfaces and combined with standard hygiene procedures could help decrease the spread of multidrug resistant bacteria in hospitals (Steindl *et al*., 2012).

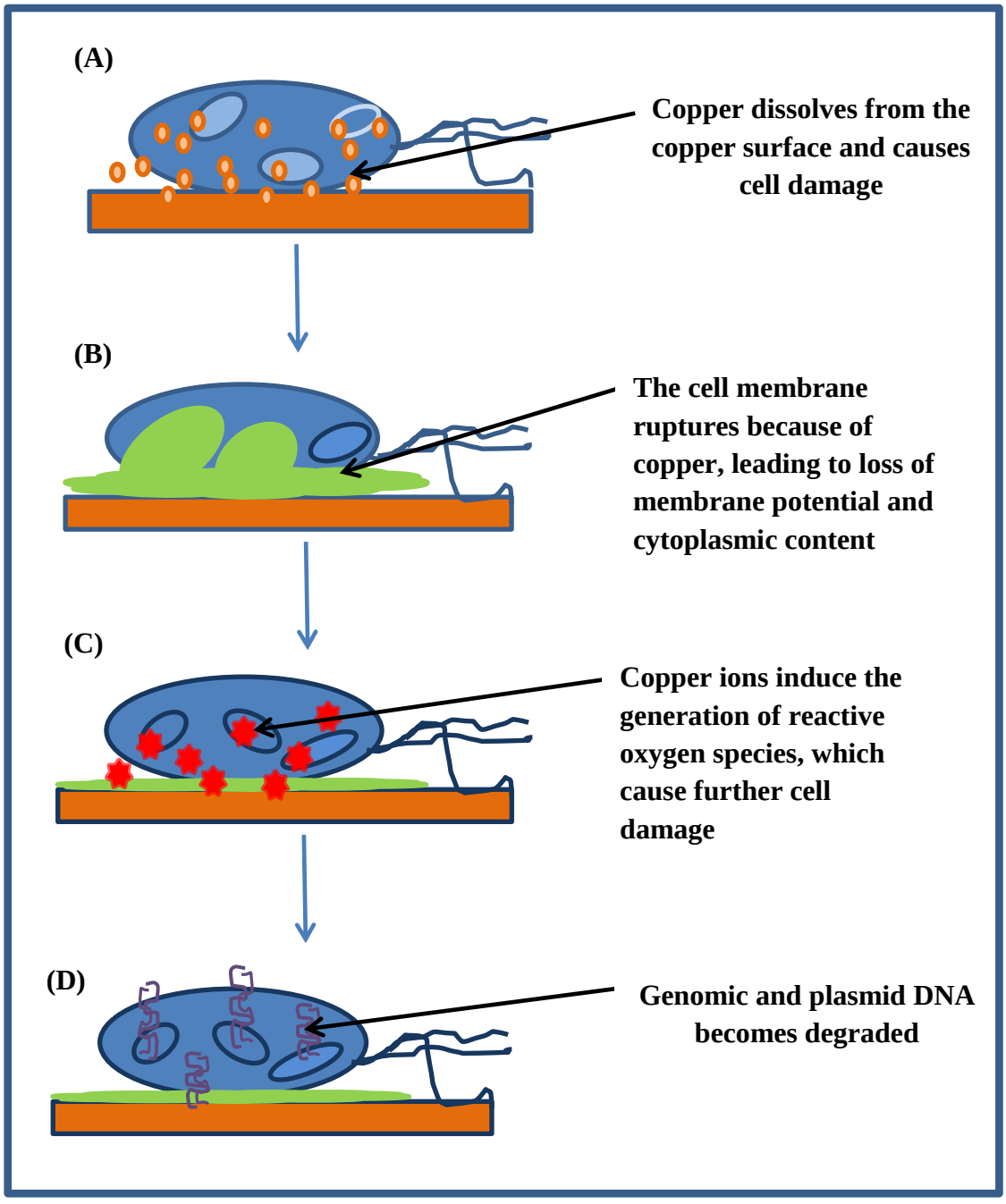

Figure 6 The action of copper ions in contact killing. Modified from (Grass *et al*., 2011).

1.6.6 Copper oxide (CuO)

There are two stable oxide phases in Cu-O systems: cupric oxide (CuO) and cuprous oxide (Cu_2O). Both are semiconductors and have band gaps in the visible or near infrared regions (Li and Mayer, 1992, Al-Kuhaili, 2008). The band gap of CuO is 1.7eV which can be activated by light with a wavelength of <approx. 720nm [equation 1]. Under aerobic conditions, superoxide [equation 2], Cu^+ [equation 3] and hydroxyl radicals [equation 4] can be produced and cause protein and DNA damage (Santo *et al*., 2008). Copper generates many reactions that produce hydroxyl radicals through Fenton and Haber-Weiss reactions (Macomber *et al*., 2007, Grass *et al*., 2011) .

$$CuO + h\upsilon(< 660\ nm) \rightarrow CuO(e^-_{cb}, h^+_{vb}) \quad [1]$$

$$CuO(e^-_{cb}) + O_2 \rightarrow CuO + O_2\bullet^- \quad [2]$$

$$Cu^{2+} + (e^-_{cb}) \rightarrow Cu^+ \quad [3]$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^\cdot \quad [4]$$

$$2Cu^+ + 2H^+ + O_2 \rightarrow 2Cu^{2+} + H_2O_2 \quad [5]$$

The hydroxyl radicals [4] can further react in a number of reactions which destroy cellular molecules by causing lipid and protein oxidation (Grass *et al*., 2011). As described below for TiO_2 copper ions can also cause depletion of sulfhydryl's such as in cysteine or glutathione [eq. 6].

$$2\ Cu^{2+} + 2\ RSH \rightarrow 2\ Cu^+ + RSSR + 2H^+ \quad [6]$$

The hydrogen peroxide produced in reaction [5] can react in reaction [4] and lead to the further generation of toxic hydroxyl radicals. However, it is still unclear what the range of reactions [2] to

[4]cause in terms of copper toxicity (Grass *et al*., 2011). Possible toxicity of copper ions is summarized graphically in Figure 7. There are a number of reports in literature supporting the fact that $^{\bullet}OH$ is powerful, non-selective oxidizing agent, and causes oxidative damage to biological molecules in cells (such as proteins and lipids) (Brandi *et al*.,1989, Cho *et al*., 2004, Dodd and Jha, 2009). However, there is not enough direct experimental data for the generation of ROS in the presence of Cu (II). In particular, there is little data about the roles of intra and extracellular ROS in the biocidal action of Cu (II). Park and others (2012) have demonstrated inactivation of *E. coli* by cupric ion (Cu II). They suggested that activation of *E. coli* was based on the reduction of Cu (II) to Cu (I) rather than the generation of ROS, and they assumed that this mechanism might be generated by all bacterial species. They showed that, in the presence of Cu (II), intracellular superoxide levels of *E. coli* decreased by increases in the concentration of copper, indicating that superoxide radical was used to reduce Cu (II) to Cu (I) in cells. Moreover, the variation amount in the hydroxyl radical level by adding Cu (II) was very small. Therefore, molecular oxygen and hydroxyl radical scavengers did not affect the inactivation efficacy of *E. coli* by Cu (II). However, it is possible that the hydroxyl radicals induced by the copper-mediated reduction of oxygen may contribute to the microbiocidal action of Cu (II) (Park *et al*., 2012).

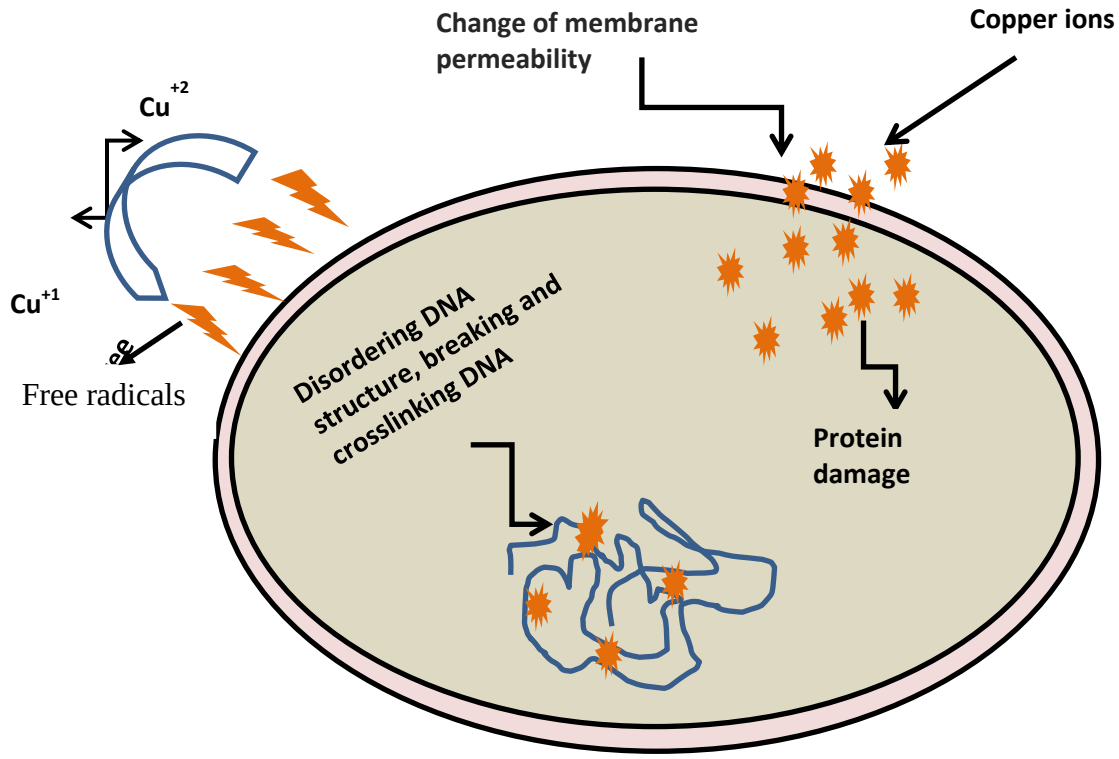

Figure 7 Mechanism of membrane disruption by copper. Modified from (Huang *et al*., 2014).

Copper oxide is the simplest member of the family of copper compounds and has many advantages; it is cheaper than silver, easily mixed with polymers and relatively stable in terms of both chemical and physical properties. CuO can also be produced in extremely high surface area form and it is valuable as an antimicrobial agent (Ren *et al*., 2009). Moreover, CuO has a non-toxic nature, the starting materials are more abundant, production costs are cheap, and there are smaller band gaps. Therefore, CuO has been used in numerous applications such as catalytic applications, electro-chromic coatings and photovoltaic materials (Al-Kuhaili, 2008). CuO has been used as a visible light activated antimicrobial coating for fabrics (Torres *et al*., 2010). CuO films are not stable in air or

vacuum at high temperatures. Therefore, copper oxide has been introduced to polymers such as silica to improve their durability and thermal stability. Silica is an absorptive substrate with a large number of surface gaps. This material was selected for CVD technology because it has a high surface area that is able to increase any chemical interactions (Norman *et al*., 1993). In addition, due to the properties including high thermal and chemical stability, biocompatibility, and high strength, hybrid silica/metals composites have many applications in medicine, physics, and chemistry. Composites of silica with different metals such as gold, titanium, platinum and aluminium have been synthesized for the catalytic applications (Kumar *et al*., 2009).

1.6.7 External target sites for copper antimicrobial activity

The initial site of copper action is thought to be plasma membrane. It has been reported that exposure of fungi, yeast, bacteria, and higher organisms to high concentrations of copper can cause selective lesions in the permeability barrier of the plasma membrane (Borkow and Gabbay, 2005). Generally, extensive copper-influenced breakdown of membrane integrity leads to loss of cell viability. However, even small modifications in the physical properties of biological membranes can cause clear changes in the activity of many essential membrane-dependent functions, including transport protein activity, phagocytosis, and ion permeability (Borkow and Gabbay, 2009). The loss of metabolic functions caused by copper ions occurs at cell membranes of both Gram-positive and Gram-negative bacteria and leads to cell death, while oxidation of susceptible components of cell cytoplasm requires more extensive oxidative degradation of the cells (Elzanowska *et al*., 1995). Many studies in the literature, including this one, have shown that Gram-negative bacteria are more sensitive to killing by copper than Gram-positive bacteria owing to cell wall differences (Liu and Yang, 2003, Dan *et al*., 2005, Elguindi *et al*., 2011). A schematic diagram of Gram-positive and Gram-negative bacterial cell walls is shown in Figure 8. The structure of the bacterial cell envelope is complex and has multiple target attack sites; some of them are present in both types of bacteria

(Gram-positive and Gram-negative). These include, the peptidoglycan layer and the phospholipid bilayer, which are present in both bacteria, but Gram-negative has two layers of phospholipid bilayer, cytoplasmic membrane and the lipopolysaccharide layer (LPS), which is present only in Gram-negative bacteria. Gram-negative bacteria possess a thin layer of peptidoglycan which makes up 10 percent of the cell wall, whereas in Gram-positive bacteria 90 percent of the cell wall is made up of peptidoglycan (Dalrymple *et al*., 2010). The thinner peptidoglycan in Gram-negative bacteria is located between the two membranes (the outer and inner membrane). The main functions of the outer membrane are protection of the bacteria from the surrounding environment by excluding toxic substances and providing an additional stabilizing layer around the cell. Lipopolysaccharides (LPS) is an important molecule since it is responsible for the endotoxic shock associated with the septicaemia caused by Gram- negative bacteria and is located in the outer membrane (Silhavy *et al*., 2010), the inner part of the outer membrane contains phospholipids. The peptidoglycan (which is bound to the outer membrane lipoprotein via peptide bond) and the outer membrane are responsible for maintaining intact cell morphology (Kiwi and Nadtochenko, 2005). On the other hand, peptidoglycan in Gram- positive bacteria is responsible for maintaining cell shape in combination with teichoic acids (TAs); peptidoglycan is a heterogeneous polymer consisting of glycan chains cross-linked by short peptides and amino acid composition. Teichoic acids are homogeneous polymers of phosphate-rich glycols that link to peptidoglycan covalently, or are anchored to the cytoplasmic membrane. Teichoic acids are present in pathogenic and non-pathogenic bacteria and are able to be involved in a variety of processes, including resistance to environmental stresses, such as heat, or cationic antibiotics and lytic enzymes produced by the host, including lysozymes (Atilano *et al*., 2010).

As previously described, copper catalyses the formation of reactive oxygen species (ROS), particularly hydroxyl radicals ($^{\bullet}OH$), via the Fenton-like reaction. The free radical produced from

Fenton-like reaction is highly reactive and capable of causing oxidative damage to cellular macromolecules (Hong *et al*., 2012). Phospholipids are the major component of plasma membrane and are one of the main targets of the ROS. So if the oxidation of plasma membrane unsaturated fatty acids is a main target of copper contact killing, membranes containing high levels of unsaturated fatty acids should demonstrate increased sensitivity to copper killing. Hong and others (2012) tested the oxidation effect of copper on unsaturated fatty acids of an *E. coli* mutant strain carrying an alteration in a gene responsible (*fabR*) for the regulation of unsaturated fatty acids biosynthesis. Loss of *fabR* leads to increases in the synthesis of unsaturated fatty acids, and consequently the unsaturated fatty level will increase in the cell membrane. They found that the increased levels of unsaturated fatty acids of *E. coli* were responsible for increasing their sensitivity to copper alloy surface killing (Hong *et al*., 2012).

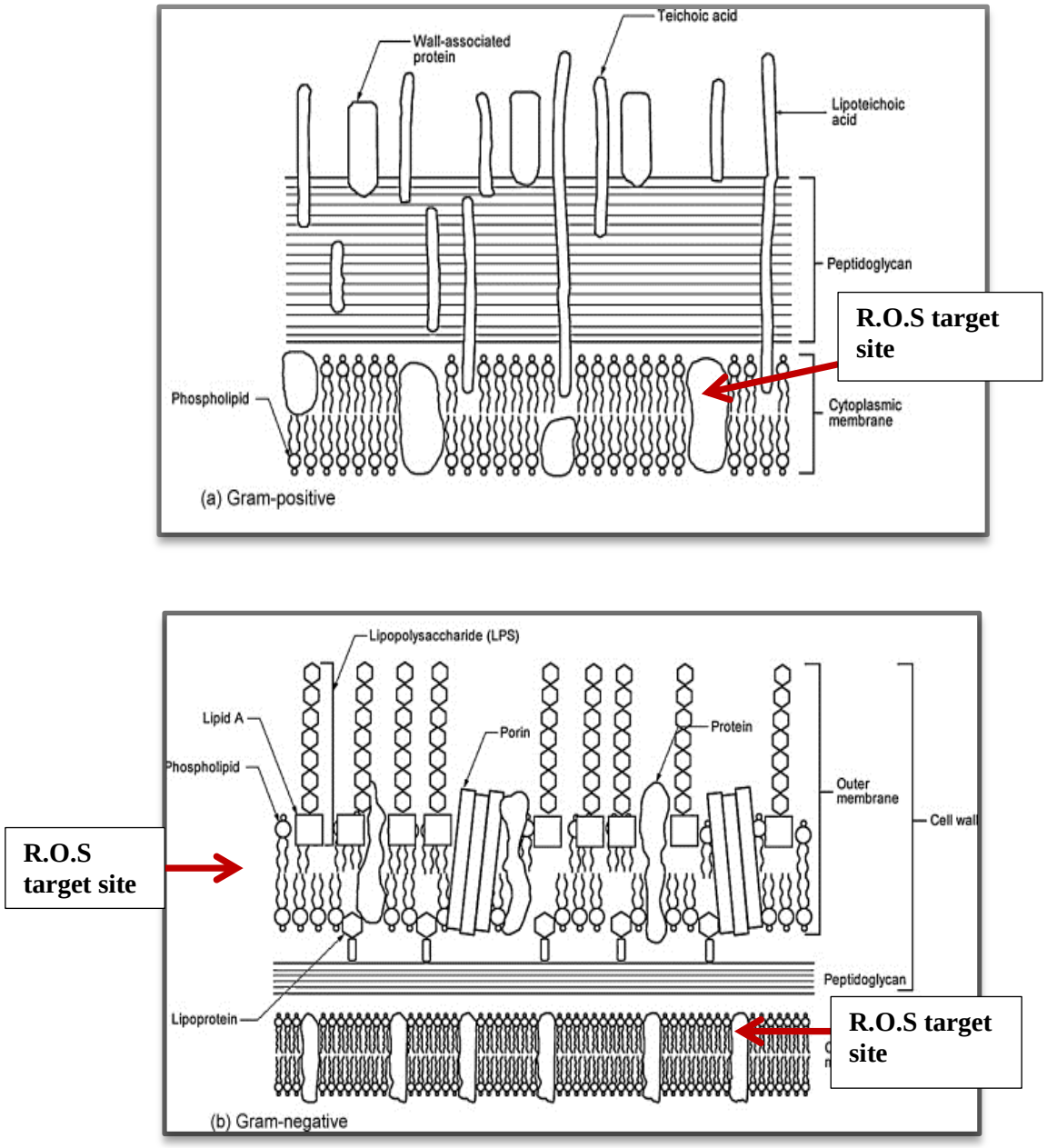

Figure 8 The structure of (a) Gram-positive and (b) Gram- negative cell envelope, arrows show the target site of R.O.S produced by copper (Dalrymple *et al*., 2010).

1.6.8 Intracellular target sites for copper antimicrobial activity

Copper ions can interact with nucleic acids by cross-linking within and between strands of DNA, which leads to their damage. Copper may cause helical structure disorders and DNA denaturation. Copper ions cause denaturation of DNA in low ionic solutions by competing with the hydrogen bonding site on the DNA molecules (Borkow and Gabbay, 2005). A study showed that the DNA double helix contains at least two different types of binding sites for copper. One site exists in every four nucleotides and has a high level of attraction for copper. The other is an introduced site for copper, which is present in every base pair. However, in single–strand DNA (such as DNA in viruses), a copper binding site was present on average in every three nucleotides with lower affinity than in double-stranded DNA (Sagripanti *et al*., 1991). Several studies have suggested that reactive oxygen species (ROS) are important in the killing mechanism during exposure to soluble copper, which can directly cause damage to nucleic acids (Lloyd and Phillips, 1999, Imlay, 2003, Harrison *et al*., 2009). In addition, it has been proposed in a recent study that in the case of *E. coli*, DNA degradation caused by copper alloys is correlated with the copper content of the alloys, and cell death occurred before DNA degradation in cells exposed to alloys containing 60 % copper. On alloys containing 99.9 % copper, complete DNA degradation occurred by 45 min and there were no shorter DNA fragments, indicating that degradation produced random-sized fragments with no preferred target sites. No loss in genomic DNA was observed in the cells exposure to 60 % copper alloys after 60 min of exposure (Hong *et al*., 2012). However, other studies on killing mechanisms of copper on *E. coli* have shown that *E. coli* growing with copper was more likely to be killed by H_2O_2 than *E. coli* growing without copper. Copper also decreased the rate of H_2O_2-induced DNA damage. So this leads the authors to suggest that copper exerts its toxicity by mechanisms other than oxidative stress (Macomber *et al*., 2007). Indeed a novel mechanism of copper toxicity was recently

demonstrated. Copper causes damage to the iron-sulphur clusters of isopropylmalate dehydratase of *E. coli*. This is one of the enzymes on the biosynthetic pathway to leucine and which has an iron-sulphur cluster that can be displaced by copper in the absence of oxygen. However, whether this mechanism is the general route of copper toxicity in bacteria is still under debate and more investigation is required (Solioz *et al*., 2010).

Copper can cause protein damage either by modifying or inhibiting biological activities. The regulation of protein tyrosine phosphatases (PTPs) is important in the investigation of the regulation of the protein tyrosine phosphorylation level, due to its role in combination with protein tyrosine of regulating cell growth, differentiation, and proliferation by controlling cellular tyrosine phosphorylation (Kim *et al*., 2000). Among different metal ions (Fe^{3+},Cu^{2+}, Zu^{+2} and Cd^{+2}) tested for their ability to inhibit human protein tyrosine phosphatase (VHR), copper was found to be the most potent deactivator. Its activity as a deactivator was about 200 times greater than that of H_2O_2, due to the oxidation of a cysteine present at the active site of VHR (Kim *et al*., 2000). It is believed that copper reacts with proteins by combining the thiol groups (-SH) of enzymes and this leads to the inactivation of the proteins (Yoon *et al*., 2007). The enzymes and protein complexes involved in the respiration chain could also be copper targets. It has been shown that MRSA had no respiring activity on copper surfaces (Weaver *et al*., 2010). Barker and others have demonstrated that oxidative stress genes are required for *S. aureus* to grow under high concentrations of copper and as protection against initial copper shock. They also predict that protein damage is an important feature of copper stress in *S. aureus*, since sudden exposure to copper causes the down regulation of *S. aureus* protein synthesis and the activation of the misfolded protein response. They showed that addition of copper to *S. aureus* resulted in a decrease in the expression of at least 20 genes encoding ribosomal proteins, translation initiation factors, and repression of two important global regulators, Agr and Sae which are essential for *S. aureus* virulence (e.g. in biofilm formation). This lead the

authors to suggest that there is a correlating decrease in protein synthesis during copper shock (Baker *et al*., 2010). Others have showed that copper ions entered the bacteria cell and caused rapid activation of CopA, which is an ATPase responsible for the transport of copper ions between cytoplasm and periplasm of *E. coli*. Thus cells were killed more rapidly (Bondarenko *et al*., 2012).

Thus, although there is a wide body of evidence supporting the antimicrobial properties of copper, there are conflicting reports about its specific mechanisms and spectrum of activity. Different methods used to produce Cu-based surface coatings; diverse inoculation methods (wet and/or dry) and different bacterial test strains have been used. Such variation in methodologies makes it hard to clearly distinguish the different effects that copper exerts on Gram-positive versus Gram-negative bacteria. In this study a wet inoculation method was used as suggested by the British standard test for antimicrobial hard surfaces (Anon, 2009b & 2011).

1.7 Titanium dioxide antimicrobials (TiO_2)

In 1972, Honda-Fujishima re-discovered the power of TiO_2, which has been known since 1921 as a photo-catalyser element. However, it is only relatively recently that it has been used in environmental cleaning such as self-cleaning tiles, glasses and windows (Zaleska, 2008). Theoretically, photocatalysis is based on the production of activated species at the surface of an irradiated semi-conductor with photons having energy higher than the one of their band gap, TiO_2 has varied band gaps ranging between three and 3.2eV, which means it can only be activated with UV-light (Puzenat, 2009). The antimicrobial activity of TiO_2 is activated when irradiated with UV radiation (wavelength <385nm). This prevents the initial adhesion of microbes, or inactivates microorganisms that adhere to a surface due to production of different reactive oxygen species (ROS). However, the half-life of most ROS is short, and they probably exist only in the region near

the catalyst surface because they can be readily suppressed in aqueous environments due to a high recombination rate. Using a photoelectron-catalytic system with an external potential bias can suppress the charge recombination (Nie *et al*., 2014). TiO_2 has been used to purify water and air in terms of environmental contamination (Sunada *et al*., 2003). The use of titania as a disinfecting agent was first proposed by Matsunaga *et al*. (1985). TiO_2 is one of the most studied materials due to its stability and photosensitivity in both powder and thin film forms (Armelao *et al*., 2007). In fact, TiO_2 surfaces are considered as promising material in future medicine, because it is not poisonous and does not cause environmental pollution. There are three different forms of TiO_2: anatase, rutile, and brookite. Rutile has a smaller band gap (3.0eV) than anatase (3.2eV), and with excitation wavelengths extends into the visible light range (410nm). Despite this, anatase is generally considered the most photo-chemically active phase of titania, due to the higher surface adsorptive capacity of anatase and its higher rate of hole trapping (see below; Visai *et al*., 2011).

1.7.1 Mechanism of titanium dioxide photocatalysis

Titanium dioxide is a semiconductor, which has band gap energy (Eg) of 3.2ev in its anatase form. The main advantage of using photocatalytic surfaces is that no electrical power or chemical reagent is required for their function, and the only ingredients required are a source of light, oxygen and water (Visai *et al*., 2011). Illuminations of TiO_2 greater or equal to Eg cause excitation of an electron from the valence band to the conduction band, leading to the formation of an electron-hole pair (Figure 9). This is a free electron in the conduction band (cb), and a hole (h^+) in the valence band (vb). These can react either within the TiO_2 itself (electron and hole recombination) or can induce the production of ROS (scheme 1) that are involved in the oxidation and reduction processes by reaction with adsorbates at the surface. This leads to degradation of the microorganism (Armelao *et al*., 2007), as a result of the oxidation of membrane lipids and the disruption of membrane

integrity, and may proceed to the complete mineralization of cellular components. These reactions are highly reactive and completely non-selective (Page *et al*., 2009).

$TiO_2 + h\nu \longrightarrow e^- + h^+$ [7]

$h^+ + H_2O \longrightarrow HO^\bullet + H^+$ [8]

$h^+ + OH^-_{(Surface)} \longrightarrow HO^\bullet$ [9]

$e^- + O_2 \longrightarrow O_2^{\bullet}$ [10]

$2O_2^{\bullet} + 2H_2O \longrightarrow 2HO^\bullet + 2OH + O_2$ [11]

Scheme 1. **Reactive radical species generated by TiO_2 photocatalysis** (Page *et al*., 2009).

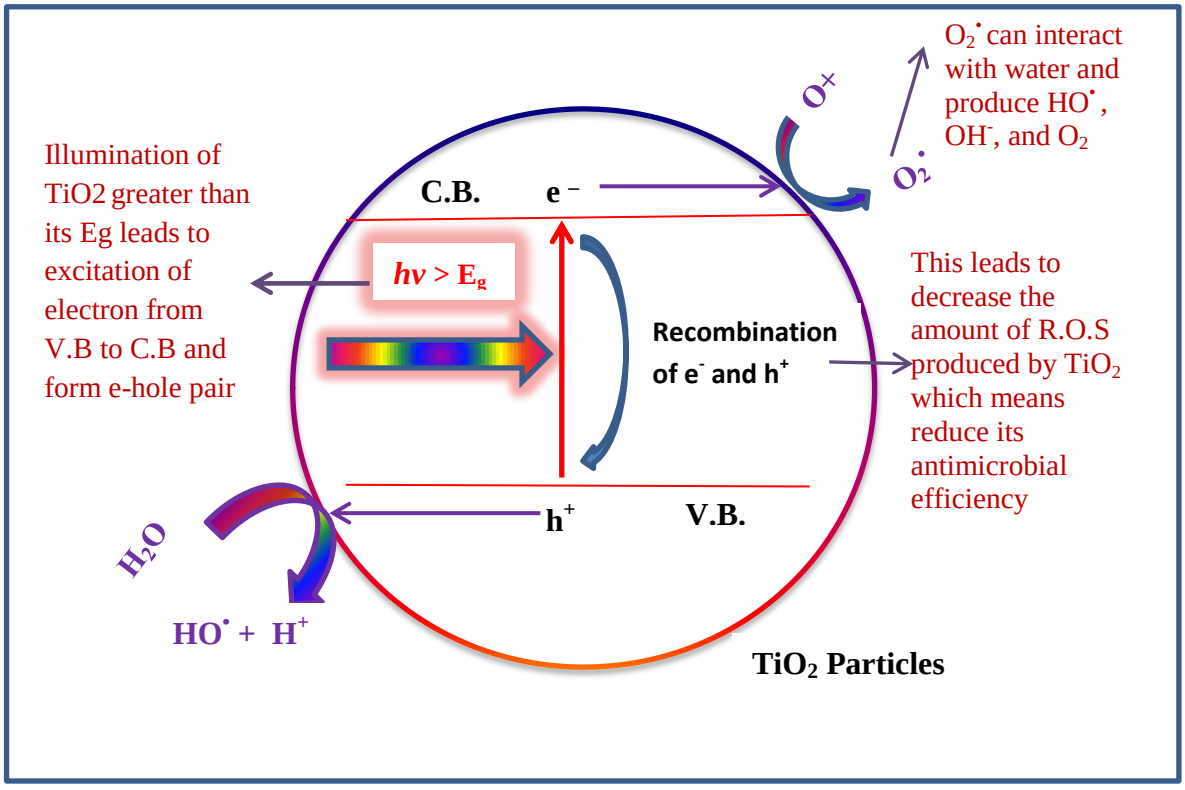

Figure 9 Processes of redox behaviour of photo-excitation in TiO_2. VB, Valence band; CB, conduction band. Modified from Page *et al*. (2009).

Many studies suggest that the toxicity of illuminated TiO_2 occur through membrane damage (Kubacka *et al*., 2014). Kiwi and Nadtochenko (2005) reported the killing of *E. coli* on TiO_2 surfaces because of membrane damage. They showed that TiO_2 caused peroxidation of three main components of the cell wall LPS (lipopolysaccharides), PE (phosphatidyl-ethanolcholine), and PGN (peptidoglycan), but the PGN was the most resistant toward peroxidation. In addition, the reaction of cell wall compound (LPS, or PE) with the hole (h^+) competes with the recombination reaction of the hole (h^+) with free electrons. This reaction is considered as the fundamental step in the radical peroxidation process (Kiwi and Nadtochenko, 2005). Maness and others (1999) demonstrate that lipids are the major targets for oxidative radical attack, and polyunsaturated fatty acids in particular

(Maness *et al*., 1999). Investigators in an early study, demonstrate cell death as a result of direct oxidation of coenzymes, which led to the decrease in respiratory activities (Matsunaga *et al*., 1985). In a recent study, bacterial membrane bound proteins were reported as the sensitive targets in the cells. The distributions of bacterial cells composition were mapped after two hours of contact with TiO_2 coated surfaces. They found that some proteins such as Amide I and Amide II signal declined in the cells compared to the healthy cells (control cells). The leakage of proteins increased as a result of the cell membrane being damaged due to increases in the permeability of the membrane (Wei *et al*., 2014) . DNA is the other possible target for the oxidative radical attack. Yand and Wang (2008) report the DNA strand breaking activity of TiO_2, which was due to active oxygen species, especially hydroxyl radical generated by UVA-irradiated TiO_2. They also show that as irradiation time increased, the photocatalytic effect on DNA also increased, and for the exposed to UV light for five seconds, the DNA damage reached 24%. However, this does not mean that microorganisms can be killed within such a short time (Yang and Wang, 2008).

It is well accepted that the bactericidal effect of photocatalysis is due to the production of reactive oxygen species (ROS) such as hydroxyl radical ($HO^{\bullet}$), superoxide ($O^{\bullet}$) and hydrogen peroxide (H_2O_2) which are generated by UV light only or by illuminated TiO_2 (Gogniat and Dukan, 2007). However, which particular radical is responsible for bacterial death is still debated (Cho *et al*., 2004). Some previous studies demonstrate that the killing of *E. coli* exposure to TiO_2 was due to H_2O_2 only because of its ability to permeate through cell membranes compared to OH. They proposed that the killing mechanism of TiO_2 is based mainly on the entering of H_2O_2 and $O^{\bullet}$ to the bacterial cell by a diffusion process and subsequently generating the $^{\bullet}OH$ through Harber-Weiss reaction. However, since only 2×10^{-7} mM of H_2O_2 was observed and this concentration falls in the range of mode I, the H_2O_2 has no killing effect (Kikuchi *et al*., 1997), the killing of *E. coli* by H_2O_2 occurred through two different modes. The first is mode I which occurs at concentrations below 2mM. However, this

concentration of H_2O_2 is not lethal and the lesions that are generated are efficiently repaired by the cells. Toxicity occurs at mode II at concentration more than 10 mM due to $^{\bullet}OH$ formed from the Fenton reaction, possibly because the repair system becomes increasingly saturated (no free valence electron) (Brandi *et al*., 1989). In fact, the $HO^{\bullet}$ generated by the above process have been considered in many studies as the main cause of the bacterial death because of the photocatalysis effect (Brandi *et al*., 1989, Cho *et al*., 2004, Dodd and Jha, 2009). Cho and others showed an excellent linear correlation between the amount of $^{\bullet}OH$ radical and the survival ratio of *E. coli* inactivation in TiO_2 photocatalysis, which indicates that the $^{\bullet}OH$ radical is the primary radical species that causes *E. coli* inactivation (Cho *et al*., 2004).

To summarize, three possible photo-killing steps of *E. coli* were proposed by Sunada *et al*. 2003. In the first step, the outer membrane is partly damaged due to changes in the membrane permeability towards reactive species. However, this does not affect the cell vitality. Cell death occurs in the second stage, when reactive species enter the cell and cause disorder of the inner membrane (cytoplasmic membrane). In the final step, decomposition of organic compounds and toxic ingredients of bacteria occurred. However, a strong UV light (1.5 mW/cm^2) is necessary to cause cell death, since the oxygen species are so active and are easily trapped at the outer membrane (Sunada *et al*., 2003).

1.7.2 Copper doped TiO_2 (Cu/TiO_2)

The efficiency of TiO_2 as a photocatalyst and an antibacterial material depends on the ability to produce electron-hole pairs with a reduced recombination rate (Armelao *et al*., 2007). This is caused mainly under irradiation by UV light. However, to improve the photocatalytic activity of TiO_2 under visible light, and to produce durable and reusable coatings, the modification of TiO_2 with metals such as Cu or Ag has been described (Page *et al*., 2009). The dual layers of Ag or Cu combined with

TiO_2 have a higher level of activity in terms of both oxidising activity and antimicrobial action than with a single layer of TiO_2 (Sunada *et al*., 2003). Indeed, the activity of TiO_2 films coated onto glass as an antibacterial and self-cleaning agent has also been proved(Yu *et al*., 2003). The antimicrobial activity of dual layers of Ag/TiO_2 and Cu/TiO_2 against different microorganisms has been repeatedly reported (Skorb *et al*., 2008, Necula *et al*., 2009, Baghriche *et al*., 2013, Wei *et al*., 2014, Yao *et al*., 2014). The effect of Cu/TiO_2 film illuminated with very weak UV light (1µ W/cm^2) on the survival of *E. coli* consisted of two steps similar to the survival on the TiO_2 films under strong UV light (1.5 Mw/cm^2) (disordering of the outer membrane followed by the disordering of the inner membrane due to the effect of ROS formed during the illumination) as represented in the schematic diagram (Figure 10). In step one, decomposition of the outer membrane occurs as a result of photocatalysis, and is followed by permeation of copper ions into the cell. In step two, loss of cell integrity occurred due to the increased copper ions penetrated into the cell, and caused cytoplasmic membrane disorder. These processes explain why the Cu/TiO_2 films show bactericidal activity under both strong and very weak UV light illumination (Sunada *et al*., 2003).

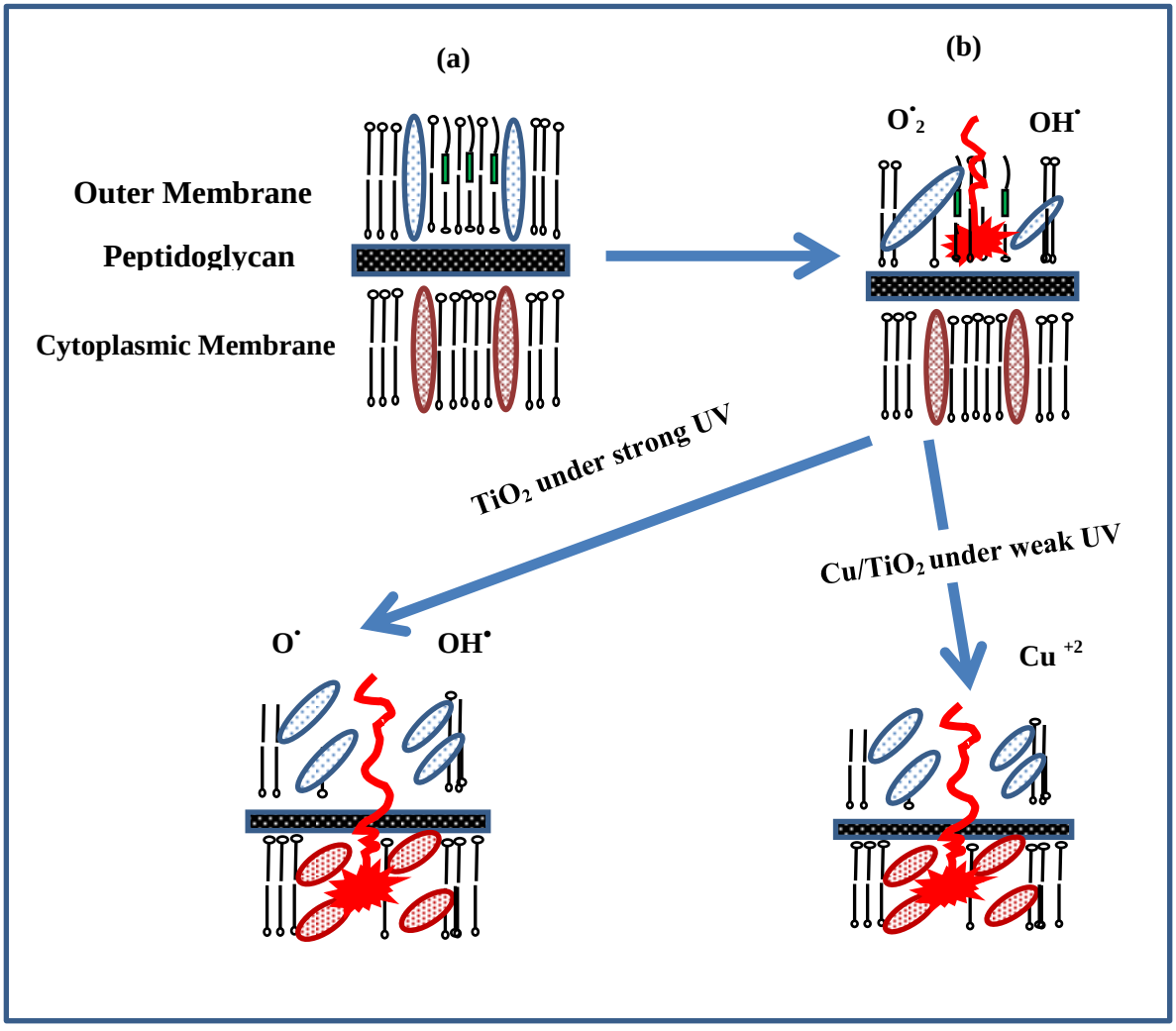

Figure 10. Schematic illustration of the bactericidal process for the copper-resistant *E. coli* cell on a normal TiO_2 film and on a Cu/TiO_2 film: (a) illustration of *E. coli* cell and (b) enlarged illustration of cell envelope exposure to TiO_2 illuminated with UV light; the outer membrane partly damaged due to R.O.S and damage of the inner membrane (c) required strong UV light (1.5 mW/cm^2) when TiO_2 film was used since R.O.S produced under weak UV (1μ W/cm^2) are easily trapped at cell surfaces. (d) Damage of the inner membrane on Cu/TiO_2 under weak UV was due to the activity of copper ions (Sunada *et al.*, 2003).

Furthermore, these films have two important properties, the first one is the photocatalytic ability to decontaminate windows by oxidizing organic stains deposited on the windows under sunlight, and

the second one is the photo-induced super-hydrophilicity which allows the removal of both organic and inorganic pollutant formed by water films (from rainfall, for instance). Moreover, two physical properties have to be addressed, the optical transparency of the glass and the mechanical resistance of the TiO_2 layer. The first one could be produced by using a very thin layer (less than 100 nm and mainly around 10 nm) and the second one could be produced by using a binder such as SiO_2 (Puzenat, 2009). A mixture of titania and silica possesses high surface areas, large pore volumes and enhanced UV photocatalytic activity compared with pure titania (Guan *et al*., 2003). Furthermore, the super-hydrophilic property of the surfaces allows the water to spread over the surfaces rather than appear as droplets. The contact angle of films consisting of TiO_2 only under UV illumination is zero, but it increased in the dark. However, films consisting of TiO_2 and SiO_2 reduce the water contact angle (which make the microbes adhere more tightly to the surfaces and thus are killed more easily) in the dark for up to 24 h (Wang *et al*., 2004).

Other relevant materials such as copper/TiO_2 include TiO_2/Cu nanosurfaces and have been used to enhance the antimicrobial activity of TiO_2 under visible light. Baghriche and others showed that nanosurfaces composed of copper and titanium have a strong effect on bacterial (*E. coli*) growth under weak visible light and in the dark. The quickest inactivation kinetics of *E. coli* (10^6) was noticed under visible light (10 min), and the killing time in the dark was longer (30 min). The different kinetics killing was due to the photo inducing, as TiO_2 when in contact with copper layers transfers the photo-induced charges to the surface Cu ions. In fact, Cu on the TiO_2 surfaces works as an electron donor and leads to faster killing. The TiO_2 able to reduce the Cu^{2+} to Cu ions by the TiO_2cb electrons, and the redox mechanism also involve the oxidizing reaction of Cu^o to Cu ions by the TiO_2vb holes. In summary, the mechanisms of enhancement due to the integrated copper are mainly due to the release of antibacterial copper ions in dark conditions, and to charge carrier separation under light irradiation (Baghriche *et al*., 2012).

Wei *et al*. (2014) prepared a new type of coating by combining Cu and TiO_2 nanoparticles into a polymer matrix (polymer-Cu/TiO_2 composites coating). This nanoparticles coating possessed excellent antimicrobial properties under sunlight, which can be used outdoors. The elimination of 10^6 of *E. coli* on the surfaces consisting of Cu only needs five hours, whereas it took two hours to kill the same amount of *E. coli* on Cu/TiO_2 coating. The hardness and wear resistance of the coating were also enhanced (Wei *et al*., 2014).

1.8 Aims and Objectives

The main purpose of this study was to investigate the antimicrobial activity of novel coated surfaces prepared by CVD.

Objectives:

- Compare the effects of exposure of bacteria to coated and uncoated surfaces.
  The antibacterial activity of coated glass surfaces containing Cu/TiO_2, Cu/SiO_2 and Ag/SiO_2; and steel copper coated surfaces were tested using British standard (BS ISO 22196:2009 & 2011) method for antimicrobial hard surfaces.
- Examine the effects of selected antimicrobial surface coatings on different bacteria including MDR pathogens of relevance in Healthcare and the food industry as well as on standard strains.
- Determine the efficiency of different types of coating
- To study factors affecting the performance of the coatings
  The effect of different levels of washing on ceramic tiles coated with Cu/SiO_2, and the effect of increased temperature (35°C) on the performance of Cu/SiO_2 and the effect of low temperature (5°C) on the performance of Cu/SiO_2 and Cu/TiO_2
- To identify the mechanisms of killing bacteria of copper/silica surfaces (Cu/SiO_2) by using Comet assay and Syto 9 staining assay.
- To develop novel methods for testing that mimic *in situ* activity
- The activity of coated ceramic tiles *in situ* (in the sluice room in Ward 37 and ward 12 at Manchester Royal Infirmary (copper coated tiles) and the activity of different coating (copper and silver coated tiles and steel and painted steel coated) placed in the ladies toilet in Salford University).

2-MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Cultures

Standard strains of *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC8739 and *Escherichia coli* ATCC 10536 were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, UK. The latter strain was used for testing photocatalytic antimicrobial activity. Different pathogenic bacterial strains of importance in HCAI and which persist in the environment including recent clinical isolates were also tested (Table 1).

Table 1 Bacterial test strains

Bacterial Species	Strain/ Isolate	Source / Reference
Gram-positive bacteria that commonly cause HCAI		
Staphylococcus aureus	ATCC 6538	*NCIMB
	EMRSA15	PHE
	MRSA 1595	PHE
	MRSA 1669	PHE
Enterococcus faecium (VRE)		PHE
Gram-negative bacteria that commonly cause HCAI		
Escherichia coli	ATCC8739	NCIMB
	ATCC10536	NCIMB
	ESBL	PHE
	ESBL2	PHE
Stenotrophomonas maltophilia		PHE
Klebsiella pneumoniae	KPC+	PHE
Acinetobacter baumannii		PHE
Gram-positive bacteria that commonly cause food-borne infection		
Listeria monocytogenes		PHE
Gram-negative bacteria that commonly cause food-borne infection		
Salmonella enterica typhimurium		PHE

*National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

PHE = Public Health England (formerly The Health Protection Agency), Manchester UK.

MRSA (Methicillin Resistant *Staphylococcus aureus*); ESBL (Extended Spectrum Beta-Lactamase producer); KPC (Carbapenemase producer)

2.1.2 Surface coatings

Different types of antimicrobial coated surfaces which included copper silica (0.25 M), silver silica (0.25 M), and copper/titanium dual layers, were prepared by flame assisted chemical vapour deposition (FACVD) by Paul Sheel and Alan Robinson, Materials and Physics Research Centre at the University of Salford) using a propane flame and burner set up (Figure 11a). The Molarity (M) refers to the concentration of precursor used. For the silica based tiles the precursor tetraethylorthosilicate was carried to the burner head using a nitrogen flow rate of 0.5 l min^{-1} from a heated and stirred bubbler (75±3ºC, stirred at 120 rpm). For copper silica, copper sulphate was used as the copper precursor and simultaneously delivered to the burner head (Figure 11b) by ultrasonically nebulising the aqueous solution into the nitrogen. For silver silica, silver nitrate was used as silver precursor and heated temperature was 95° C± 3ºC. The glass substrate was passed through the flame on a translating stage several times (routinely 6 times) to give a coating of approx. 25 nm. Substrate temperature was 190ºC. The glass was cut into 2 cm squares and the inactive side of the glass substrate was marked by scratching the surface at the top edge of a glass with a diamond tipped pencil. Samples were then disinfected by shaking in 70% aqueous methanol for 20 min and air dried before use. Uncoated float glass was used as a control. Concentration of metal precursors was usually 0.25M. The same method used to coat the glass with Cu/SiO_2 was also used to produce coated 15 cm^2 white glazed ceramic tiles (Kai Group, Bulgaria obtained from B & Q Ltd, UK). However, because of the size differences between coater head and tiles; a strip 10 cm wide was coated down the centre of the tile. All sampling (swabbing) was done from this coated area.

Production of copper/titanium dual layers coatings was by a two-step process. The Cu/CuO films were grown on glass (1 mm borosilicate glass) using an Atmospheric pressure- Flame assisted chemical vapour deposition (AP-FACVD) coater. In this process, flame energy is used to decompose

the CVD precursors and a film grows when this is directed at a substrate. Substrate heating was employed, which enhanced film density and adhesion. The substrate temperature was set at 300°C. An aqueous solution of 0.5 M Cu $(NO_3)_2$ was nebulized into a carrier of N_2 at a rate of 2 dm^3 min^{-1} through the flame and onto the substrate with a propane/oxygen flame ratio of 1:20. The films were removed from the reactor and allowed to cool before subsequent reheating to 580 °C for TiO_2 deposition. The thermal CVD films were deposited using a custom-built APCVD reactor. The precursor for the TiO_2 deposition was titanium tetra-isopropoxide (TTIP; 107 °C, 1.0 dm^3 min^{-1}) transported to the reactor via a bubbler into 6.5 dm^3 min^{-1} process gas.

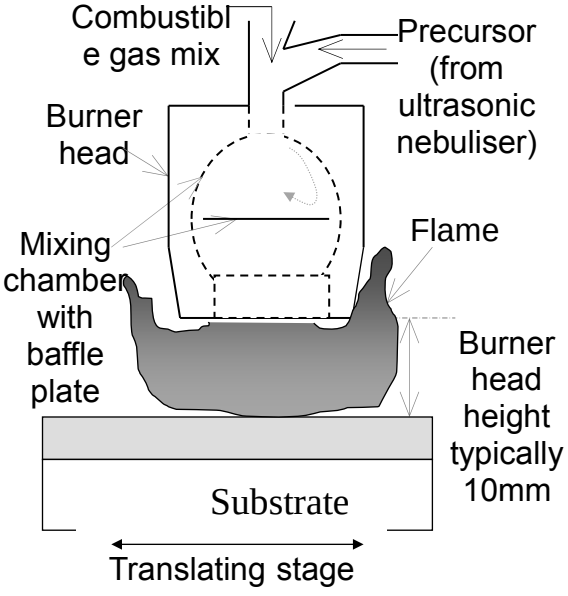

Figure 11 a Set-up for Chemical Vapour Deposition

Taken from (Cook et al., 2011)

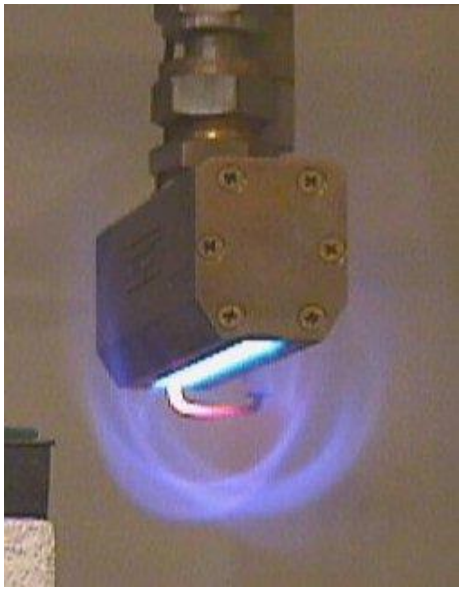

Figure 11 b Chemical Vapour Deposition .Burner head showing propane flame. Taken from (Cook et al., 2011)

OCAS samples (5 samples, table 2) were also prepared by the above method (FACVD) and commercial stainless steel (type 304- supplied by Aperam) was used as coated substrate instead of glass or ceramic tile. Steel was coated with a thin (multilayer) coating. Two types of CVD layers were deposited: pure silica films (reference silica) and multilayer films, consisting of silica base layer, an intermediate copper oxide layer and a silica top-layer. The combustion gases were propane and air. Hexamethyldisiloxane (HMDSO) and copper nitrate Cu $(NO3)_2$ were used as precursors for silica and copper oxide.

Table 2 Preparation of OCAS samples

Samples Code	**Number of steps under flame**		
	Base layer (Silica)	**Intermediate layer (Copper)**	**Top layer (Silica)**
2	**5**	**20**	**5**
2b	**5**	**20**	**5**
2c	**5**	**40**	**5**
5	**10**	**40**	**10**
Ref silica	**10**	**-**	**-**

2.2 Methods

2.2.1 Microorganisms and growth conditions

Bacterial strains were sub-cultured onto Nutrient Agar (NA, Oxoid, Basingstoke, UK) and incubated at 37ºC for 24 h. Cultures were resuspended in Nutrient Broth (NB, Oxoid) and kept on Microban® beads (TCS Ltd., Merseyside, UK) at - 80ºC. Prior to use, one bead was sub-cultured onto NA and incubated at 37ºC for 24 h.

2.2.2 Antibacterial activity test of Cu/SiO_2 and Ag/SiO_2 (in the dark)

Antibacterial activities of coated surfaces (Cu/SiO_2 and Ag/SiO_2) were determined according to the method described in BS ISO 22196:2009 & 2011(Anon, 2009b, Anon, 2011) with modifications (glass cover slips were used rather than plastic film and the incubation was at room temperature rather than 35ºC). Colonies were re-suspended in a 1:500 dilution of NB and adjusted to O.D 0.01-0.02 at 600 nm in a spectrophotometer (Camspec, M330, Cambridge,UK) to give approx. $2x10^6$ colony forming units (CFU) cm^{-3}. Fifty microlitres was inoculated on to each 2 cm square test sample (inactive side down, so that only the active surface was tested) and control samples (un-coated glass), and covered with a square of pre-sterilised 1 mm borosilicate glass to ensure close contact between the culture and the film. The samples were housed within 50 mm diam. Petri dishes (to minimise contamination from the laboratory environment); containing moistened sterile filter paper to prevent drying out of the suspensions. Samples were incubated at room temperature (25°C) and removed after 0, 1, 2, 4, 6 and 24 h and immersed aseptically in 20 cm^3 of Tryptone Soy Broth (Oxoid, CM0129) and vortexed for 60 sec to re-suspend the bacteria. A viability count was performed by dilution and plating on Tryptone Soy Agar (TSA, Oxoid, CM0131) in triplicate and incubation at 37ºC for up to 48 h.

2.2.3 The effect of fluorescent light on antibacterial activity of Cu/SiO_2 glass coated surface

The effect of fluorescent light on the antibacterial activity of Cu/SiO_2 was determined according to the method described in BS ISO 22196:2009 & 2011 (Anon, 2009b, Anon, 2011) as described above in step 2.2.2. However, inoculated substrates were irradiated with Philips daylight fluorescent lamps (UVA 0.01 mW cm^{-2}). Standard *Escherichia coli* (ATCC8739) was used for this test.

2.2.4 The effect of illumination (UV and fluorescent light) on antibacterial activity of glass coated surface (Cu/TiO_2).

Antibacterial activity of dual layer $CuO\text{-}TiO_2$ coatings was determined according to the method described in BS ISO 27447:2009 (Anon, 2009a) which is same procedure as described above in step 2. However, the samples were irradiated with Backlight Blue lamps with a maximum UVA light intensity of 0.24 mW cm^{-2} as shown in figure 12 (lamp chamber, LS-15 UVI Ltd, Cambridge, UK). Control samples were covered with a polylaminar UVA protection film (Anglia Window films, UK) to protect samples from UVA light. Philips daylight fluorescent lamps (UVA 0.01 mW cm^{-2}) were used for some experiments. Temperature of samples was approx. 25°C during illumination.

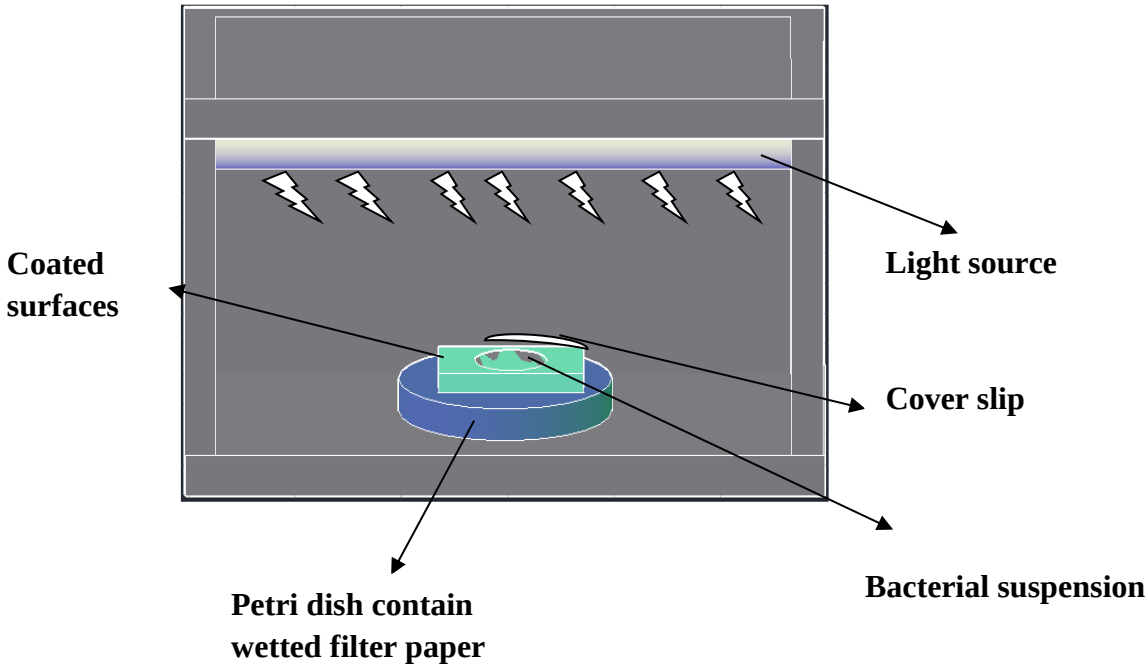

Figure 12 Illumination chambers for UV or/and fluorescent light

2.2.5 The effect of different temperature (35 °C and 5°C) on antibacterial activities of Cu/SiO_2 and Cu/TiO_2.

The procedures described in steps 2.2.2 and for Cu/SiO_2 were followed; however coated surfaces were incubated in Petri dishes containing ice to yield a temperature of approx 5°C during the experiment. And for 35°C samples were incubated at approx 35°C incubator during the experiment. Standard *S. aureus* (ATCC6538), standard *E. coli* (ATCC8739), and MRSA 15 were used for 35°C test on Cu/SiO_2 coated glass. For the 5°C test, ESBL producing *E.coli* and *A. baumannii* were used for both surfaces Cu/SiO_2 and Cu/TiO_2.

A summary of all tested bacterial strains on different coated surfaces is shown in Tables 3, 4 and 5.

Table 3 Bacterial strains tested at 25° C on Cu/SiO_2, Ag/SiO_2, Cu/TiO_2 and OCAS samples

Coating type	Bacterial strains tested at 25°C		
	Dark test	**UV test**	**Visible light test**
Cu/SiO_2	*E. coli* (ATCC 8739**)** including ESBL producing strains		Standard test strain of *E. coli* (ATCC 8739)
	Acinetobacter baumannii		
	Klebsiella pneumoniae		
	Salmonella enterica Typhimrium		
	Stenotrophomonas maltophilia		
	Enterococcus faecium (VRE)		
	Listeria monocytogens		
	Staphylococcus aureus (ATCC6538) included MRSA strains		
Ag/SiO_2 (0.25M)	Standard *S. aureus* (ATCC6538)		
	Klebsiella pneumoniae		
Ag/SiO_2 (0.05M)	*Acinetobacter baumannii*		
	Stenotrophomonas maltophilia		
Cu/TiO_2	ESBL producing *E. coli*	ESBL producing *E. coli*	ESBL producing *E. coli*
		Standard *E. coli* (ATCC 10536)	*A .baumannii*
		A. baumannii	
		MRSA1595	
		Klebsiella pneumoniae	
		Enterococcus faecium (VRE)	
OCAS (Steel samples; 2, 2b, 2b-1,2C and 5)	Standard *S. aureus* (ATCC6538)		
	Salmonella enterica Typhimrium		
OCAS (painted steel; 2,2b,2b-1, 2C and 5)	Standard *S. aureus* (ATCC6538)		
	Standard *E. coli* (ATCC 8739**)**		
	Salmonella enterica Typhimrium		

Table 4 Bacteria strains tested at 5°C on Cu/SiO_2 and Cu/TiO_2

Coating type	Bacteria strains tested at 5°C		
	Dark test	**UV test**	**Visible light test**
Cu/SiO_2	Standard test *E. coli* (ATCC 8739)		
	Standard test *S. aureus* (ATCC6538)		
	A. baumannii		
	ESBL producing *E. coli*		
Cu/TiO_2		ESBL producing *E. coli*	ESBL producing *E. coli*
		A. baumannii	*A. baumannii*

Table 5 Bacteria strains tested at 35°C on Cu/SiO_2

Coating type	Bacteria strains tested at 35°C
	Dark Test
Cu/SiO_2	Standard *E. coli* (ATCC 8739)
	Standard *S. aureus* (ATCC6538)
	MRSA15

2.2.6 Statistics

Where possible each experiment was done in triplicate and viable counts were determined by calculating means, standard deviations and T-tests using Microsoft Excel. Survival curves were plotted (using Sigma plot software) as the means with standard deviations as error bars. In order to allow plotting survival curves on a logarithmic scale, because zero cannot be plotted on a logarithmic scale, one was added to each mean viable count. In some cases error bars were obscured by the graph symbols and in others only upper error bars were plotted.

2.2.7 Effects of inoculum size

To determine the effect of inoculum size on the time required for total kill on copper-silica surfaces (Cu/SiO_2), different concentration of bacteria suspension (*E. coli* ATCC 8739) were used. Cu/SiO_2 coated glass samples were tested with different inocula 10^3 and 10^5 CFU ml^{-1} following the method described in section 2.2.2.

2.2.8 Confirmation that bacteria were removed from the coated surfaces

E. coli (ATCC 8739) was used for this experiment. Samples were inoculated on surfaces as described above (step 2.2.2). Following re-suspension the samples were removed aseptically from the TSB and stained with crystal violet (1% aqueous) then observed under a light microscope.

2.2.9 Determination of minimum inhibitory concentration of Copper (MIC)

Two different bacterial strains were tested. These included standard *E. coli* (ATCC 8739) and standard *S. aureus* (ATCC6538). Bacterial cultures were sub-cultured as described above in step 2.2.1. 10^6 of bacteria concentration was used by measuring the optical density of 0.01– 0.02 using spectrophotometer at 600 nm.

1 $mgml^{-1}$ of copper sulphate Cu $(SO_4)_2$ was used as copper precursor.

One hundred microliters of Iso-Sensitest broth (Oxoid, CM0473 ,UK) was add to the all wells on the micro-titer plate (Sterillin Ltd,612F96, UK) and serial dilutions were set up by loading 100µl of copper sulphate to the first well (1 mg/ml), mixing and transferring 100 µl to the second well (0.5mg/ml). This technique was repeated across the plate to the well 10 which gives series dilution of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01625, 0.008, 0.004 and 0.002 mg ml^{-1} (Table 6). 10µl of bacteria solution was then loaded to the wells 1 – 10. Wells 11 and 12 were positive and negative controls. The positive control (11) consisted of 100µl of growth medium and 10µl of bacterial

solution, and the negative control (12) consisted of 100µl of growth medium and 10µl of sterilized water. Plates were incubated at 37°C for 4h. Plates were examined using microtitre plate reader at 570 nm (Multiskan FC microplate photometer, Thermo Scientific, UK) to determine the 4 hour MIC and a loop full of each well was taken and streaked across Tryptone Soy Agar plates which incubated further for 20 h. the lowest concentration showing no growth after 4h was recorded as the 4^{th} hour MIC. The microtitre plates were then further incubated at 37°C for 24h and again examined using microtitre plate reader to confirm the 24 h MIC (since the lowest concentration that had growth after 4 h may have been killed after extra incubation time).

Table 6 Shows the conversion of dilution values from mg/ml to mM

Mg/ml	mM
1	4
0.5	2
0.25	1
0.125	0.5
0.06	0.25
0.03	0.125
0.015	0.06
0.008	0.03
0.004	0.015
0.002	0.008

2.2.10 Antimicrobial activity of coated surface in real life use

Antimicrobial activity of the coatings on tiles (Cu/SiO_2) was confirmed by cutting the coated section of the tiles into 2 cm^2 samples. These were tested by the same method as the coated glass BS ISO 22196:2011(Anon. 2011) as previously described above in step 2.2.2 except that the sterilization was performed by autoclaving at 121°C for 15 min. Standard *E. coli* (ATCC 8739) was used for this experiment.

2.2.10.1 *In situ* activity in a ladies toilet facility in Salford University

In order to investigate the performance of the coated tiles in "in use" situations, test and control coated samples were mounted on board and exposed to natural contamination in the ladies toilets in the Peel Building of the University of Salford (Figure 13, Table 7). Substrates were wiped with 70% ethanol prior to starting the experiment to remove any contamination that had occurred while handling. The board with the samples was turned 180 degrees 3 times per week on alternate days. The tiles were left in place for 2 weeks and sampled by swabbing an area (7.5 x 5 cm for tiles, 5.5 x 9 cm for glass samples, 5.5 x 6.9 cm for steel samples) using a template. Swabbing was repeated after 2, 9, 14 and 28 weeks. Because of high levels of contamination the samples were washed after 28 weeks using a 10g l^{-1} solution of Tergitol™ and allowed to dry. Samples were taken after 30, 32, 41, 46, 50, 54, 63, 68, 73, 79 and 85 weeks with careful washing with detergent solution followed by rinsing with sterile distilled water after 42 and 64 weeks.

Table 7 Key for samples in toilet facility

CVD, CVD Technologies Ltd, Manchester, UK. OCAS NV, Zelzate, Belgium.

Sample number	Coating	Substrate	Made by
1, 2	Ag- SiO_2	Tile	CVD
3, 4	control	Tile	CVD
5, 6	Cu -SiO_2	Tile	CVD
9	Blanco (control)	Painted steel	OCAS
10	2 + Cu/SiO_2	Painted steel	OCAS
11	2b + Cu/SiO_2	Painted steel	OCAS
12	2b1 + Cu/SiO_2	Painted steel	OCAS
13	2c + Cu/SiO_2	Painted steel	OCAS
14	ref 5 silica	Painted steel	OCAS
15	5 + Cu/SiO_2	Painted steel	OCAS
16	Blanco (control)	Steel	OCAS
17	2 + Cu/SiO_2	Steel	OCAS
18	2b + Cu/SiO_2	Steel	OCAS
19	2b1 + Cu/SiO_2	Steel	OCAS
20	2c + Cu/SiO_2	Steel	OCAS
21	5 + Cu/SiO_2	Steel	OCAS
22	ref 5-silica	Steel	OCAS
23, 24	Cu-SiO_2	Glass	CVD
25	control	Glass	CVD

Figure 13 Shows substrates mounted on board located in lades toilet in Salford University.

2.2.10.2 At Manchester Royal Infirmary (Gastrointestinal Surgery ward 37 and Renal Sluice Room ward 12).

Coated surfaces of Cu-SiO_2 ceramic tiles were glued on the wall next to the sink and the bench in the sluice room in wards 37 and 12 as shown in Figures 14 and 15, and subjected to the normal cleaning regime (washing with Chloroclean™ disinfectant 0.1% chlorine approx. twice weekly). The coated and uncoated tiles (10 x 15 cm) were left in place for 5 months and sampled by swabbing as described below. Swabbing was repeated after 20, 28, 36, 44 and 72 weeks. This experiment was originally designed to be by swabbing monthly but due to difficulties in gaining access to the hospital, swabbing was not done every time and, for ward 12, the tiles were removed and broken up "accidentally" by a cleaner after the second sampling and thus the work continued with just the tiles on Ward 37.

Figure 14 Antimicrobial activity of coated surface (Cu-SiO_2 Ceramic Tiles) located in sluice room at Manchester Royal Infirmary (Gastrointestinal Surgery ward 37)

Figure 15 Antimicrobial activity of coated surface (Cu-SiO_2 Ceramic Tiles) located in sluice room at Manchester Royal Infirmary (Renal Sluice Room ward 12)

- **Swabbing protocol (Standard procedure provided by the Health Protection Agency (now Health Protection England)**

Swabs used were Medical Wire NRS™ Transwab® containing 5 ml Neutralising Buffer. Swabs were moistened in the buffer and applied 15 times horizontally and further 15 times vertically in zig zag pattern over the surface so that the entire area was sampled. Swab was rotated while sampling. Each swab was immediately transferred to the neutralising buffer tube and closed. In the lab the swabs were vortexed for 60 sec and serially diluted using saline. One hundred microlitres from each dilution was inoculated onto Tryptone Soy Agar plates in triplicate. The colony forming units were counted after 24 h. In some experiments selective media were used for isolating 'Indicator Organisms' these included Baird-Parker Agar (Oxoid, CM 0275) for staphylococci, Kanamycin Aesculin Azide Agar (Oxoid, CM0591)for enterococci and Chromocult Coliform Agar (Merck, VM141650010) for coliforms.

2.2.11 Surface Morphology

The morphology of surfaces was investigated using Scanning Electron Microscopy (SEM; Philips XL30) with samples sputter coated with a 2-3 nm layer of Pt/Pd (platinum/palladium) to provide a conductive surface.

2.2.12 Determination of film density

Transmission of the coatings in visible light was measured using an Aquilla NKD7000 spectrometer using plane polarized light source and transmission averaged over 400-700 nm and measured at a 30° angle.

2.2.13 Durability of (Cu/SiO_2) coating

2.2.13.1 Tape test

Scotch tape testing was performed by applying pressure-sensitive tape to an area of the coating which was cross hatched every 5 mm with a diamond scribe. The adhesive tape was then applied and pressed firmly to ensure consistent contact with the coating. Adhesion was deemed acceptable if no film was pulled off by the tape when it was removed. Samples were observed visually and then under a microscope to determine if the integrity of the film had been maintained.

2.2.13.2 Scratch test

To assess the hardness of the deposited coatings, films were scratch tested using a constant load scratch hardness tester (Figure 16). A diamond tipped scribe was moved through 50 mm over the surface with a 100 g load. The mean width of the resulting scratch over 6 points was then measured under 200x optical magnification and compared to similar data from materials of known Mohs

hardness (aluminium, steel, copper, glass and quartz) and Mohs hardness values of the deposited films were calculated. Results are the means of three determinations.

Figure 16 Scratch tester machine (made by the Material and Physics Centre at Salford University)

2.2.13.3 Washability test

The scratch resistance and the effects of different cleaning levels (low, medium, high) on coated tiles (copper tiles) were investigated to evaluate the durability and any effects of cleaning on the biocidal activity of surfaces. Twelve Cu-SiO_2 coated tiles were sent to OCAS, Belgium, 4 for each cleaning level and then returned for antibacterial activity testing.

- **Washability method:**

This test was performed by OCAS, Belgium, they developed equipment to evaluate the washability, brushability and wear resistance of inks, paints and coatings as shown in figure 17. The test tool (e.g. normal or abrasive sponge) was placed in a tool holder, which moved back and forth over the test sample. The speed, displacement length and cycles can be adjusted and a weight can be applied. additional load of 335g.

Figure 17 Washability test equipment

The copper content of the 12 samples were measured throughout the washability test by taking mean of X-ray fluorescence (XRF) in order to determine the amount of Cu reduction after each washing step. The machine used was XRF XEPOS (energy dispersive polarised X-ray fluorescence spectrometer for multi-element analysis). XRF intensities were translated to elemental concentrations by means of calibration lines for each individual element obtained for standard materials (using Turbo Quant Alloys). Preliminary experiments showed that even 2000 rubs with a soft sponge only removed approx 28% of the copper. Conditions were therefore developed using an abrasive sponge. Three different washing procedures of increasing intensity termed low, medium and high wash were used. The amount of copper removed was 75% after the high wash. Conditions were as following:

1- **Low wash** (test 1):

100 rubbing cycles with abrasive sponge without additional load.

2- **Medium wash** (test 2):

Same as test (1) plus 500 additional rubbing cycles with abrasive sponge and additional load of 335g.

3- **High wash** (test 3):

Same as test (2) plus 400 additional rubbing cycles with abrasive sponge and

- **Antibacterial activity testing after washing**

A modification of the standard method was developed to allow testing of larger samples. Tiles were wiped with 70% ethanol prior to starting the experiment to eliminate any contamination that had occurred while handling. 9 tiles were used 3 tiles for each wash cycle. Each tile was divided into four equal sections and marked (each section was 5 cm by 2.5 cm). For each test (low, medium, high) two control tiles had been used, one was plain glass and the other one was an unwashed copper coated tile. The tiles were then sprayed with 0.1 ml overnight culture of *E. coli* (standard laboratory strain ATCC6538) using a spray gun (Fisher Scientific, 215-020S, UK) and placed in a Petri dish (245 mm, BD Falcon Biodish, 351040) on top of sterile filter paper (Fisher Scientific, FB 59015) with 3 ml sterile water to prevent drying out. Samples were kept in the dark. Samples were swabbed at 0, 4, 6, and 24 h using a swab (Medical Wire MW 774). Each swab was immediately transferred to the 5 ml neutralising buffer tube which was closed then vortexed for 60 sec. Suspensions were then serially diluted using saline and100 µl from each dilution was inoculated onto Tryptone Soy Agar plates in triplicate. The colony forming units were counted after 24 h. Each tile was used once and the results were mean of three experiments.

2.2.14 Mechanism of Killing of copper (Cu/SiO_2)

In ordered to investigate the killing mechanism of copper two different experiments were conducted. Membrane damage was tested using the Live/dead staining procedure and DNA damage was determined using the Comet assay. The bacteria tested were standard *E. coli* (ATCC 8739) and standard *S. aureus* (ATCC6538).

2.2.14.1 Determination of membrane integrity

In this assay a mixture of SYTO 9 green fluorescent stain and propiodium iodide red fluorescent stain was used (live/dead BacLight Kit L-7007, Invitrogen, UK). The SYTO 9 stain labels bacteria with both intact and damaged membranes. However, propidium iodide only stains bacterial with damaged membranes. Cultures were inoculated on Cu-SiO_2 surfaces as described above (step 2.2.2), 50 µl of bacteria were aseptically removed after different times (0, 1, 2, 4, 6, and 24 h) and stained using the live and dead staining procedure provided with the kit. Bacterial cells were suspended in 200 µl of the staining mixture which was prepared by mixing 3 µl of SYTO 9 and 3 µl of PI in 1 ml of filter sterilized water. After staining samples were kept in dark for 15-20 min and observed under a fluorescent microscope (Dialux 20 EB, DFC310 FX camera, Leitz UK).

2.2.14.2 Comet assay

A comet, or single-cell gel electrophoresis (SCGE), assay was generally carried out as described by Trevigen (Comet Assay K IT, 4250-050-K, TREVIGEN Instructions, USA). This assay was performed with standard *E. coli* and standard *S. aureus*. Briefly, bacterial suspensions (10^6 cfu ml^{-1}) were prepared as described above (step 2.2.2), however in this experiment bacteria were suspended in PBS (Ca^{++} and Mg^{++} free) after different incubation times on Cu-SiO_2 coated surfaces. Bacterial cells were removed and transferred into a cuvette. For fixation, cells were mixed with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and 50 µl immediately pipetted onto CometSlide™. Slides were placed flat at 4° in the dark for 10-15 min. Further steps were performed at 4°C and ice-cold reagents were used to minimize DNA damage. The samples were immersed in 4°C Lysis Solution (4250-050-01, Trevigen,USA) for 30-60 min in the dark to avoid denaturation of DNA by light (lysis solution acts to remove cell membrane and cytoplasm). The excess buffer was drained from slides and then immersed in freshly prepared Alkaline Unwinding Solution (PH>13 200 mM NaOH, 1mM EDTA) for

20-60 min at room temperature in the dark (the unwinding solution allow any breaks in DNA single and/or double sites to migrate towards anode when exposed to current during electrophoresis producing tail or comet like fragment). Slides were carefully placed in the electrophoresis slide tray in an electrophoresis tank and 950 ml of pre chilled Alkaline Electrophoresis Solution (8 g NaOH pellets and 2 ml of 500mM EDTA pH 8) was added. Slides were subject to 35 v for 40 min. Slides were gently removed and immersed twice in distilled H_2O for 5 min each, then immersed in 70% ethanol for 5 min. Samples were kept overnight at room temperature to dry out and then stained by SYBR Green solution (1 µl of SYBR Gold and 10 ml of TE Buffer, pH7.5; TE: 10mM Tris-HCL pH 7.5, 1mM EDTA), 100 µl of SYBR Green solution was added onto each sample and placed in refrigerator for 5 min. Slides were kept to dry in dark at room temperature and then viewed by fluorescent microscope (Dialux 20 EB, DFC310 FX camera, Leitz, UK).

Chapter 3

Antimicrobial activity of coated surfaces against standard test strains of bacteria and hospital pathogens

3.1 Introduction

Despite their low cost and high ability to inhibit bacterial growth, organic antibacterial materials are not currently used. There are a number of reasons for this. Firstly, their antibacterial effects only last for a short time. Secondly, organic compound pollutants have generated various problems in living conditions, public health and industrial fields. Therefore, to solve these problems, new antibacterial materials have been developed and studied. Inorganic antibacterial materials containing antibacterial metals like silver or copper, with the properties of longevity, stability, safety and broad spectrum antibiosis, may overcome the drawbacks of organic antibacterial materials (Jing *et al*., 2008). Silver has been known for a long time as an antibacterial agent against a wide variety of microorganisms and has been used safely in medicine for many years. It has been reported that stainless steel gained excellent antibacterial properties when implanted with copper ions and kept its corrosion resistance (Jing *et al*., 2008). TiO_2 is a disinfectant which has been used to eliminate or kill microorganisms in water, in air, and on surfaces. However, to increase their photocatalytic antibacterial efficiency, TiO_2 was deposited with metal elements such as copper or silver to decrease their band gap energy, which enables it to respond to the visible light spectrum. These kinds of coatings were able to kill both Gram-positive and gram-negative bacteria and viruses (Foster *et al*., 2010). It is widely known that copper has strong inhibitory effects on a wide variety of microorganisms. Currently, the effect of copper on bacteria, in particular bacteria that cause the majority of hospital-acquired infections such as MRSA and *ESBL E. coli*, has been widely studied (Zhang *et al*., 2012a).

In this study, coated surfaces were prepared using CVD technique since it has advantages compared to other methods as described in Chapter 1. A variety of coatings were prepared initially and are described in Chapter 3, this chapter presents the results of the killing activity of the surfaces on different bacteria chosen because they are important causes of HCAI, persist in the environment or are important food-borne pathogens. Because the Cu/SiO_2 coatings gave good results and, the ease of their preparation and availability of test samples, these were used for the majority of the tests including *in situ* testing (Chapter 5) together with 12 coated samples prepared by chemical vapour deposition and obtained from OCAS. The latter were only tested with the food borne pathogens as the main interest by OCAS is in food-related applications.

3.2 Results

3.2.1 Antimicrobial activity of Ag/SiO_2 (coated glass) at room temperature (25°C)

Glass samples coated with Ag/SiO_2 were tested for activity against bacteria using the modified BS ISO 22196:2009 & 2011 method. Because the hardness of the Ag/SiO_2 (0.25 M) coating was low compared to other materials tested, a lower concentration (0.05 M) of Ag was also tested which had a higher scratch resistance (Cook *et al*., 2011) . The coated surface with the higher concentration of Ag (0.25 M) was highly active against the Gram-negatives with a >5 log reduction (99.999%) within 4h for *K. pneumoniae* and a 3.2 log reduction in 24 h for the Gram-positive *S. aureus* 6538 (methicillin sensitive strain used for disinfectant testing) whereas there was only a small reduction on the control glass (Figure 18). However, the activity was lower with the lower concentration of Ag. The activity of low Ag coating against *A. baumannii* and *S. maltophilia* is shown in Figure 19. *S. maltophilia* was most resistant with only 1.3 log reduction after 6 h increasing to 1.8 log reductions after 24 h, whereas *A. baumannii* showed 2 log reductions after 6 h increasing to 2.5 log reduction after 24 h. Despite the fact that the samples with high concentration of Ag was softer than the

samples with a lower concentration, the difference in test results of 0.25M coating was significant for both strains for *S. aureus* and *K. pneumoniae* ($P < 0.001$ and $P < 0.05$ respectively) compared to control surfaces after 24 h.

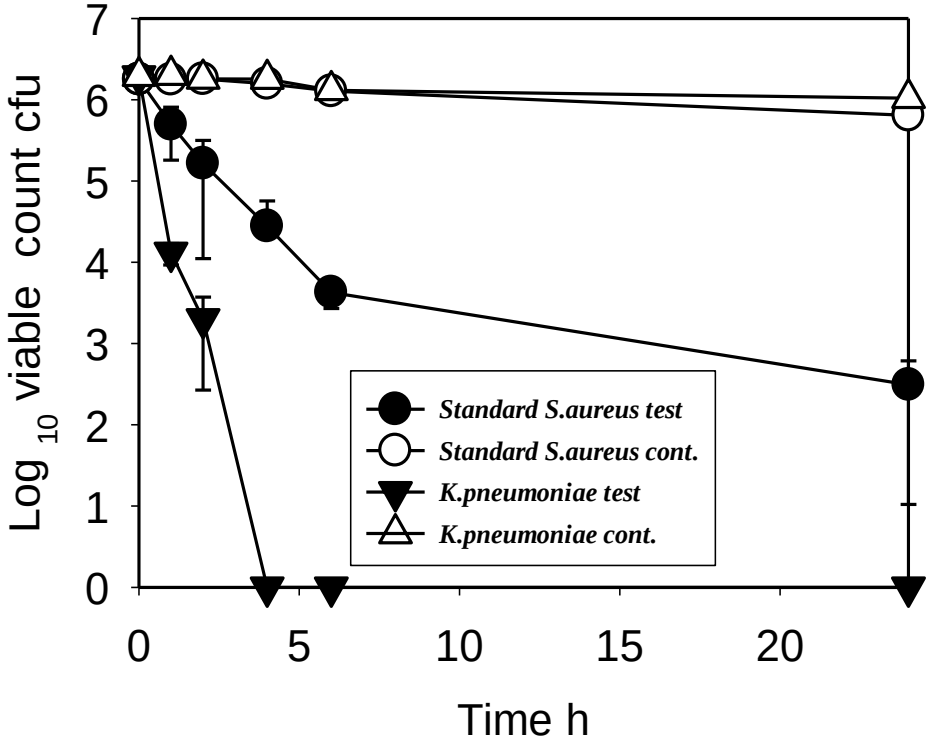

Figure 18 Killing of standard *S. aureus* (ATCC 6538) and *K. pneumoniae* on Ag/SiO_2 coated glass (0.25 M -low hardness) and uncoated glass (control) at room temp. (25°C).

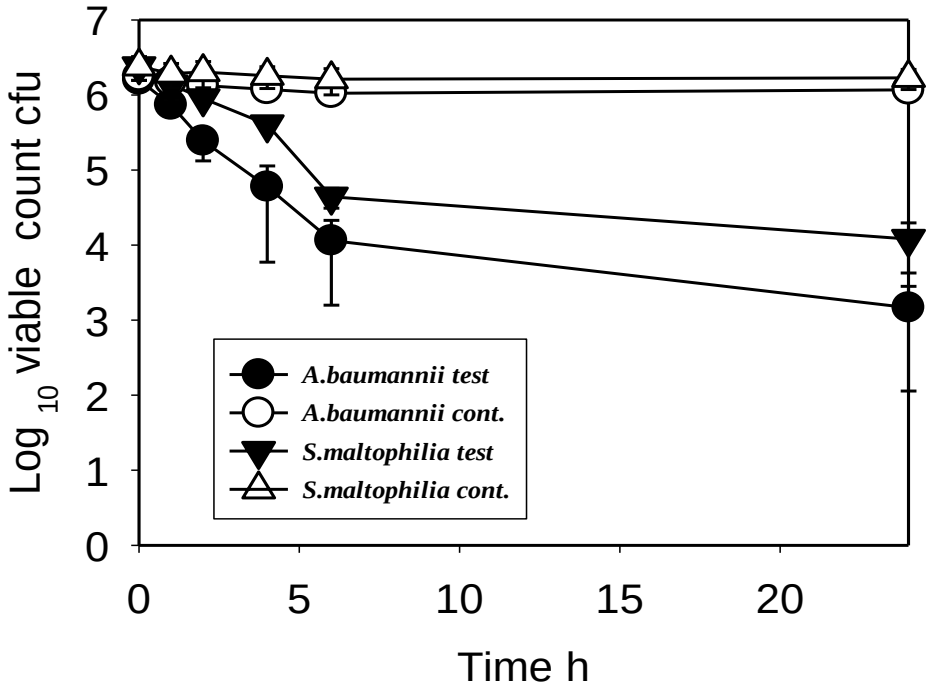

Figure 19 Killing of *Stenotrophomonas maltophilia* and *Acinetobacter baumannii* on Ag/SiO_2 coated glass (0.05 M test) and uncoated glass (control) at room temp. (25°C).

3.2.2 Antimicrobial activity of Cu/TiO_2

3.2.2.1 The antimicrobial activity of Cu/TiO_2 under UV light at room temperature (25°C).

The activity of Cu/TiO_2 coated against an Extended spectrum β-lactamase producing ($ESBL^+$) strain of *E. coli* and standard test strain of *E. coli* (ATCC 10536) are shown in Figure 20. The standard test strain of *E. coli* were more sensitive to killing on Cu/TiO_2 under UV light than $ESBL^+$*E. coli* with a >5 log kill after 6 h compared to a >5 log kill after 4 h for the standard test strain of *E. coli*. *A. baumannii* was as sensitive as standard test strain with a >5 log kill after 4h; whereas the killing curve of *K. pneumoniae,* VRE (Gram-positive) were broadly similar to $ESBL^+$*E. coli* with a >5 log

kill after 6 h as shown in Figure 21. MRSA1595 was more resistant with only a 2.3 log reduction in viability after 6 h. However this still represents a 99% reduction and a >5 log kill was obtained after 24 h (Figure 22).

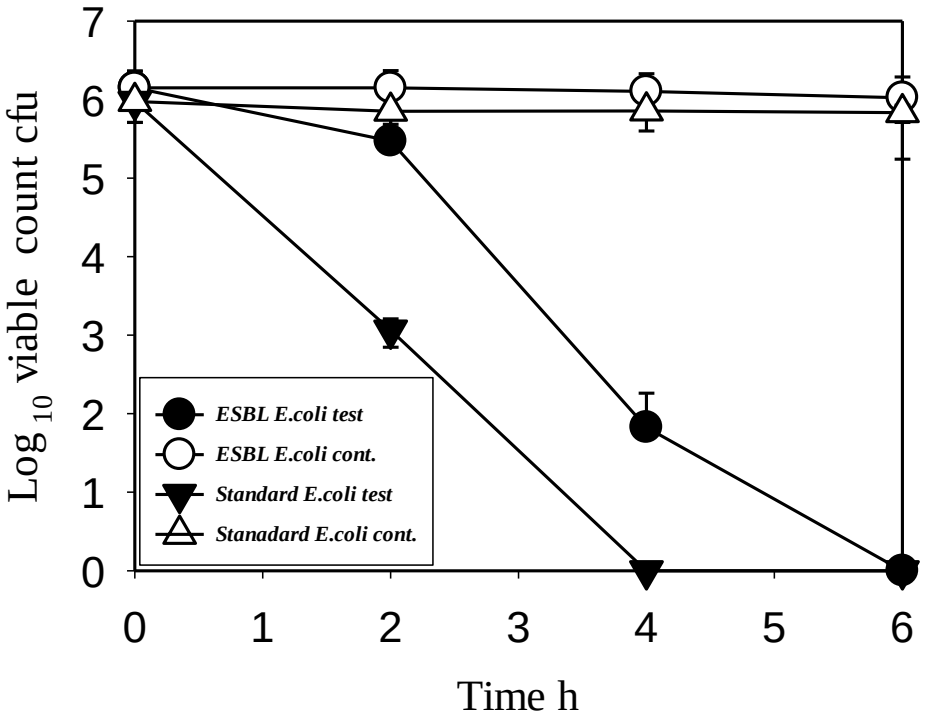

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Figure 20 Killing of ESBL *E. coli* and standard *E. coli* (ATCC 10536) on Cu /TiO_2 coated glass (test) and uncoated glass (control) under UV light at 25°C (room temp.)

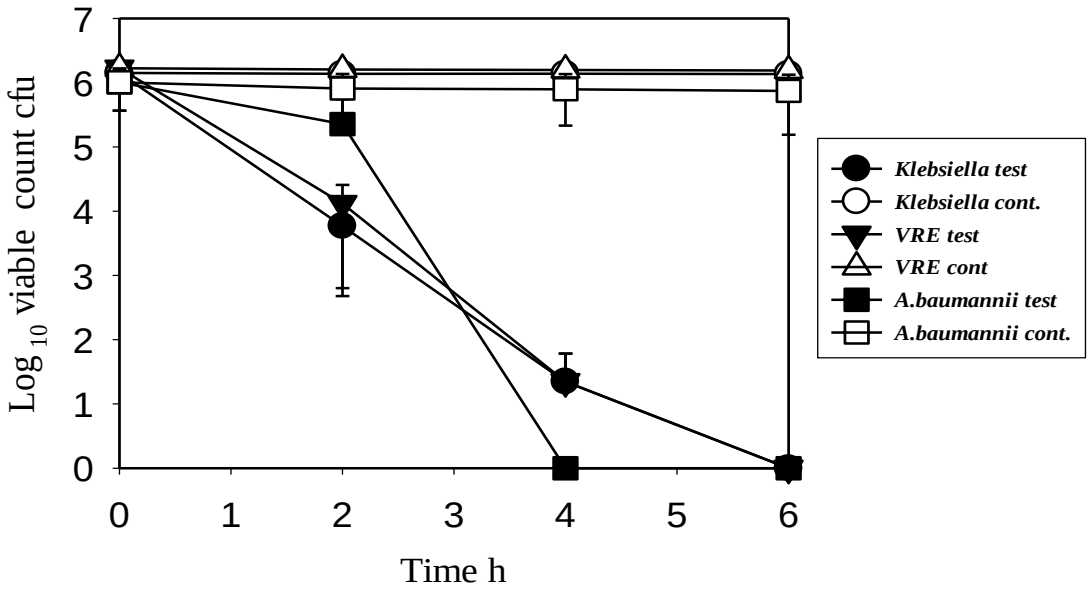

Figure 21 Killing of *A. baumannii,* VRE and *K. pneumonia* on Cu /TiO_2 coated glass (test) and uncoated glass (control) under UV light at 25°C (room temp.).

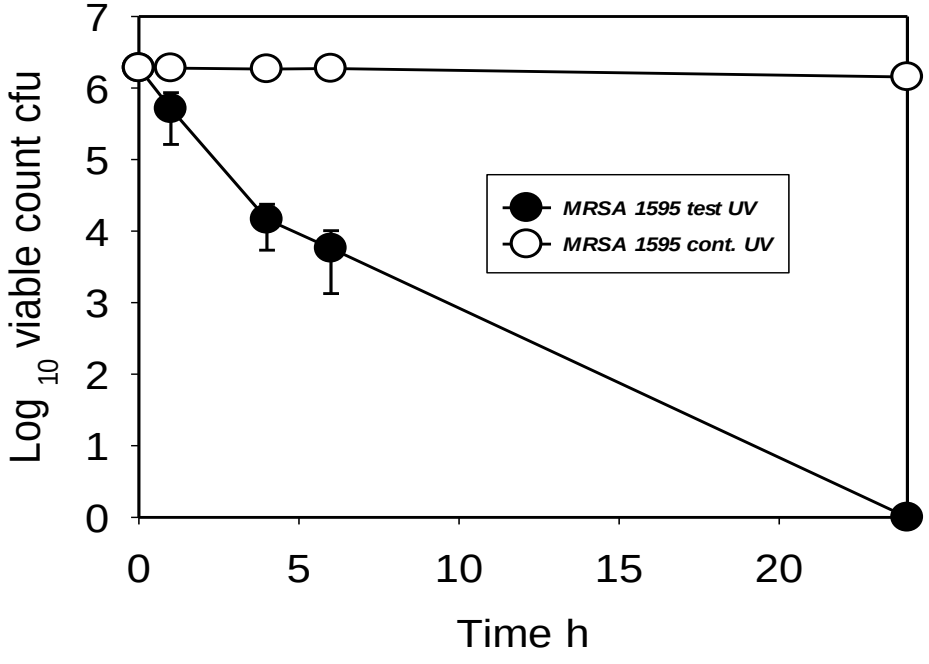

Figure 22 Killing of MRSA1595 on Cu/TiO_2 coated glass (test) and uncoated glass (control) under UV light at 25°C (room temp.).

3.2.2.2 The antimicrobial activity of Cu/TiO_2 under florescent light and Dark at room temperature (25°C).

The activity of Cu/TiO_2 against ESBL *E. coli* in the dark and under florescent light is shown in Figure 23. The ESBL *E. coli* was more sensitive when UVA ($P < 0.05$) was used for irradiation with 4.3 log reduction after 4 h compared to 2.5 log reduction when visible light was used for irradiation ($P \geq 0.05$) and only 1.5 log reduction in the dark ($P \geq 0.05$). However, after 6 h more than 5 log reductions of ESBL *E. coli* was seen under all conditions been tested (UV, Fluorescent and dark). Dark activity of Cu/TiO_2 was probably due to Cu activity. *A. baumannii* was also more sensitive to killing by UVA than visible light with a >5 log kill after 4 h under UVA and only 2 log under visible light which increased to >5 log after 6 h as shown in Figure 24.

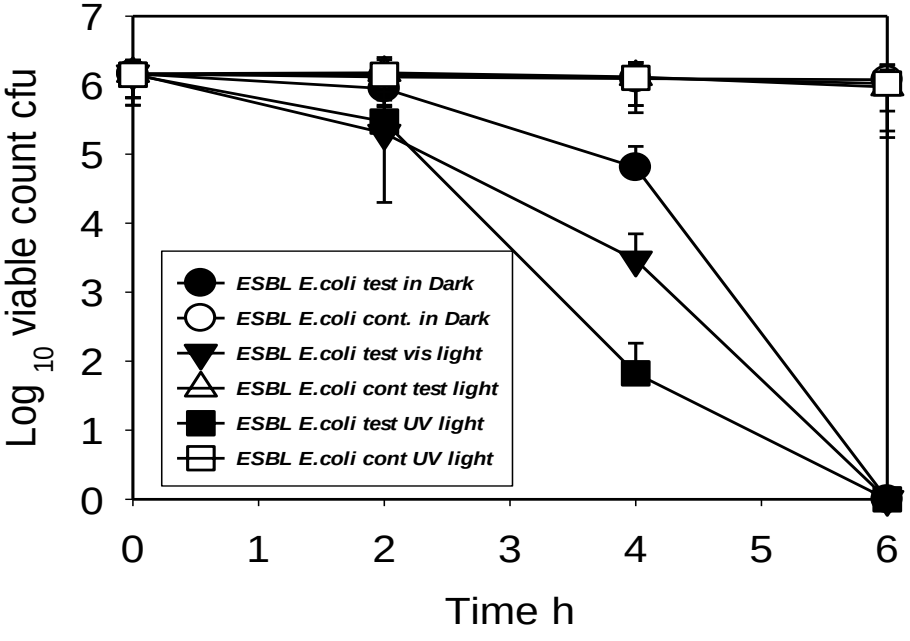

Figure 23 Killing ESBL *E. coli* of under different illumination (UVA, fluorescent light and dark) on Cu/TiO_2 coated glass (test) and uncoated glass (control) at 25°C (room temp.).

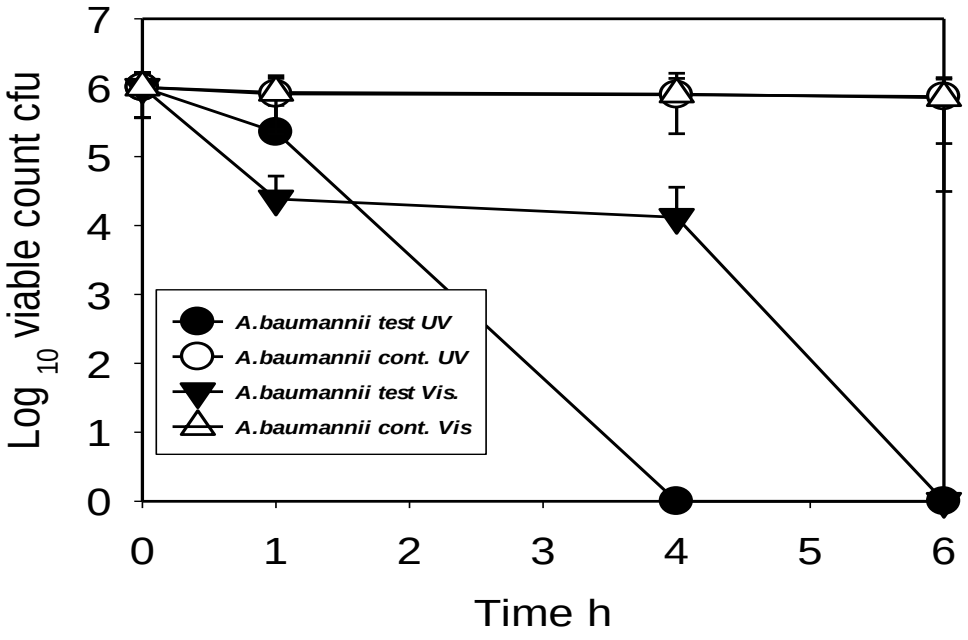

Figure 24 Killing of *A. baumannii* under different illumination (UVA and fluorescent light) on Cu/TiO_2 coated glass (test) and uncoated glass (control) at 25°C (room temp.).

3.2.3 Antimicrobial activity of Cu/SiO_2 (coated glass) at room temperature (25°C) in the dark.

Glass samples coated with Cu/SiO_2 were tested for activity against bacteria using the modified BS ISO 22196:2009 & 2011 method. The activity of the Cu/SiO_2 against the standard test strain of the Gram-negative *E. coli* (ATCC8739) and the Gram-positive *S. aureus* ATCC6538 (methicillin sensitive strain used for disinfectant testing) is shown in Figure 25. The coated surface was very active against the *E. coli* with a > 5 log reduction (99.999%) within 4 h for *E. coli* and a 4 log reduction (99.99%) for the *S. aureus* in 24 h whereas there was only a small reduction on the control glass (Figure 25). A complete kill for *S. aureus* was seen after 48 h (data not shown). The films were equally active against the Kpc^+ (carbapenemase producing) *K. pneumoniae* and again gave a >5 log reduction after 4 h (Figure 26). The activity of the Cu/SiO_2 against *A. baumannii* and *S. maltophilia* are shown on figures 27and 28 respectively. *A. baumannii* and *S. maltophilia* had a similar

sensitivity to *E. coli* but the ESBL and ESBL-2 producing *E. coli* strains and *S. enterica Typhimurium* were more resistant with only a 2.5 log kill for ESBL *E. coli* strains and 2 log for *S. enterica Typhimurium* after 6 h increasing to >5 log kill after 24 h (Figures 27, 28, 30). Recent MRSA strains (1595 and 1669) isolates were more resistant compared to other organisms tested with only an approx. 2 log kill after 6 h increasing to 2.8 log and 3.3 log kill after 24 h respectively but the differences between tests and controls were still significant after 24 h (with *P* value 0.001 and 0.0006, Figure 29). VRE and *Listeria* were also highly resistant with only 1 and 1.2 log reduction after 6 h increasing to 2.8 and 2.5 log reductions respectively after 24 h (Figure 26 & 30). MRSA 15 was the most resistant of organisms tested with only 1.2 log kill after 6 h (Figure 29).

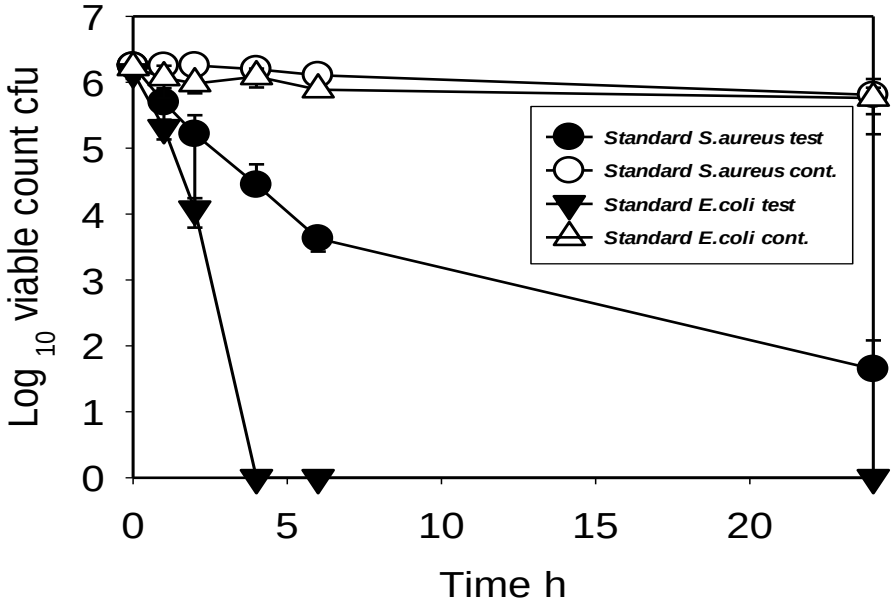

Figure 25 Killing of standard *E. coli* (ATCC8739) and standard *S. aureus* (ATCC6538) on Cu/SiO_2 coated glass (test) and uncoated glass (control).

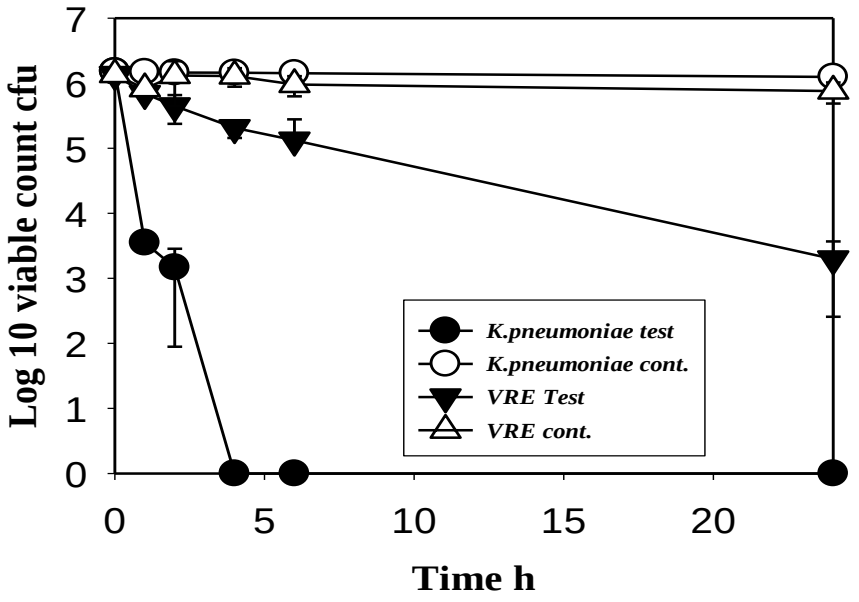

Figure 26 Killing of *K. pneumoniae* and VRE on Cu/SiO_2 glass (test) and un-coated glass (control).

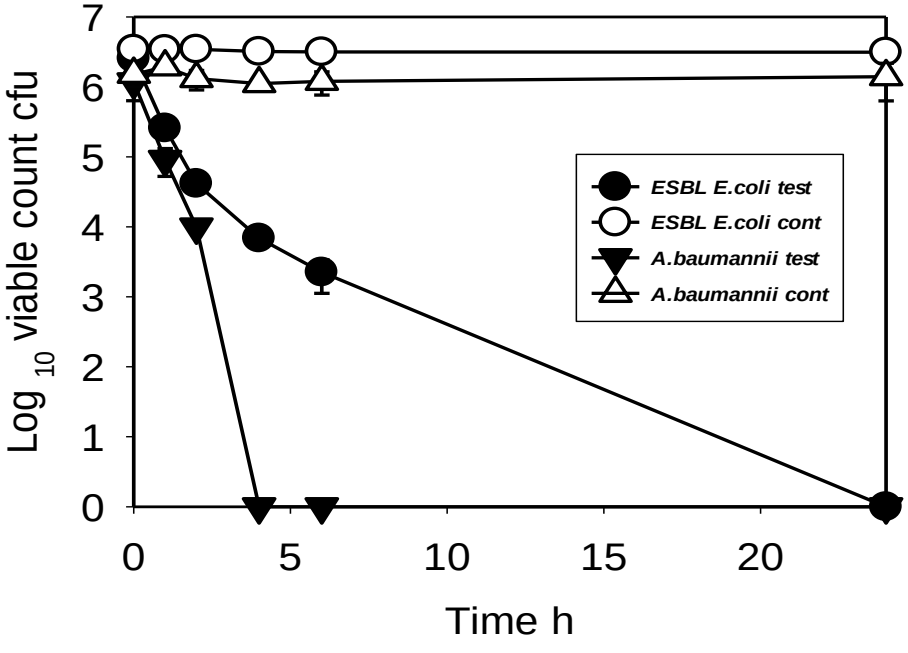

Figure 27 Killing of ESBL *E. coli* and *A. baumannii* on Cu/SiO_2 coated glass (test) and un-coated glass (control).

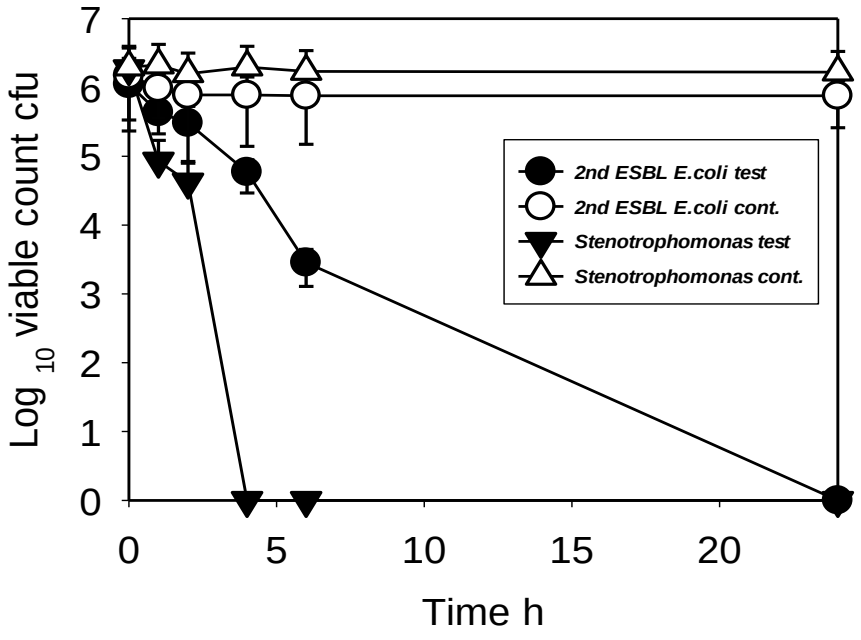

Figure 28 Killing of ESBL-2 *E. coli* and *S. maltophilia* on Cu/SiO_2 coated glass (test) and un-coated glass (control).

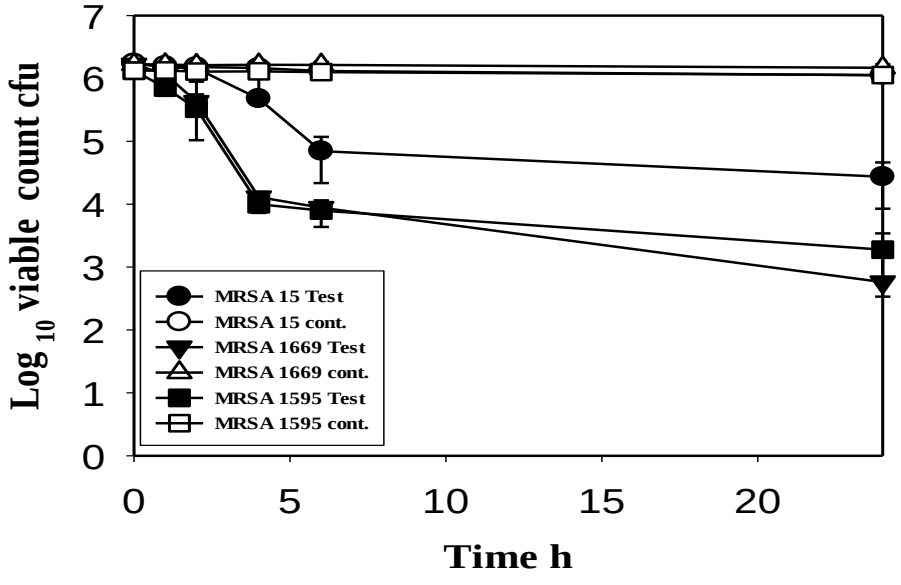

Figure 29 Killing of MRSA 15 MRSA 1595 and MRSA 1669 on Cu/SiO_2 coated glass (test) and un-coated glass (control).

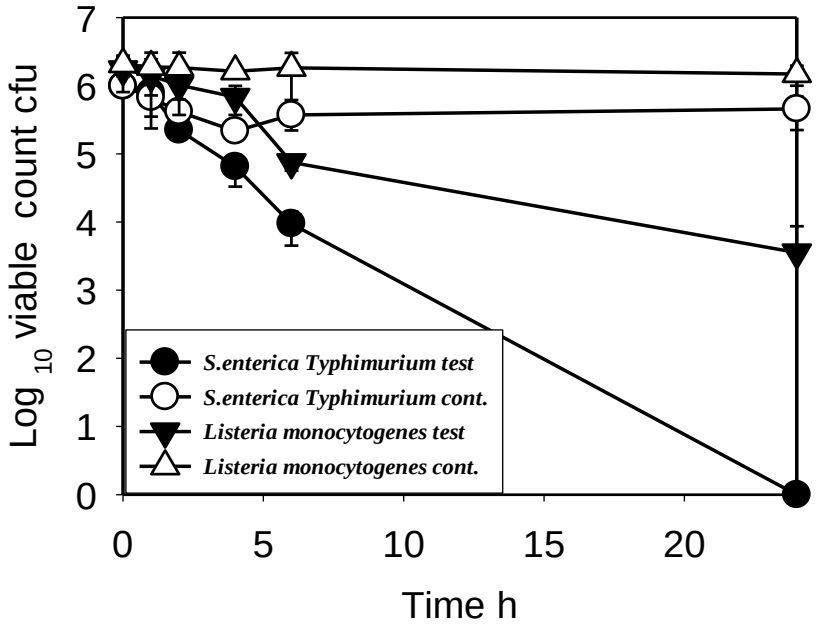

Figure 30 Killing of *Listeria monocytogenes* and *S. enterica Typhimurium* on Cu/SiO_2 coated glass (test) and uncoated glass (control).

3.2.3.1 Effects of cell loading on antimicrobial activity of Cu/SiO_2 against standard *E. coli* (ATCC8739) at 25°C

The effects of cell loading were studied by determining the activity of Cu/SiO_2 against different concentrations of standard *E. coli* strain and the results are shown in Figure 31. Concentrations of *E. coli* of 10^6 and 10^5 which were equivalent to 2.4×10^5 and 3.6×10^3cfu cm^{-2} showed similar killing curves with 2 log reductions after 2 h which increased to 5 log reductions after 4 h. It is possible that the 5 log reduction for the 10^5 cfu ml^{-1} was achieved after 3 h but intermediate times were not tested. However at the lowest concentration tested (10^3, equivalent to 2.2×10^2 cfu ml^{-1}) complete killing was reached within 2 h (99.999%). The rate of killing was similar for all three concentrations.

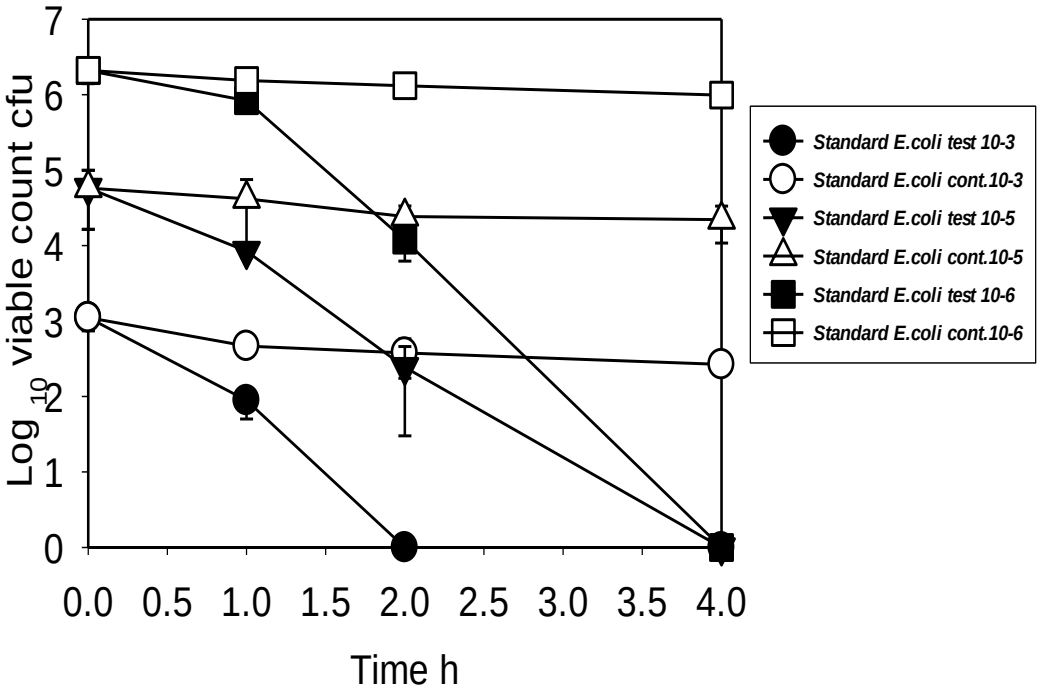

Figure 31 Killing of different concentration of standard *E. coli* (ATCC8739) on Cu/SiO_2 coated glass (test) and uncoated glass (control) at room temperature (25°C).

3.2.3.2 The effect of fluorescent light on antimicrobial activity of Cu/SiO_2

The activity of Cu/SiO_2 on the standard *E. coli* strain under fluorescent light is shown in Figure 32. The coating surface was more active under fluorescent light with > 5 log reduction after 2 h (P = 0.0017) under fluorescent light compared to a >5log after 4 h in the dark (P = 0.03) compared to the controls. The increased activity was probably due to the small fraction of UVA emitted from fluorescent light.

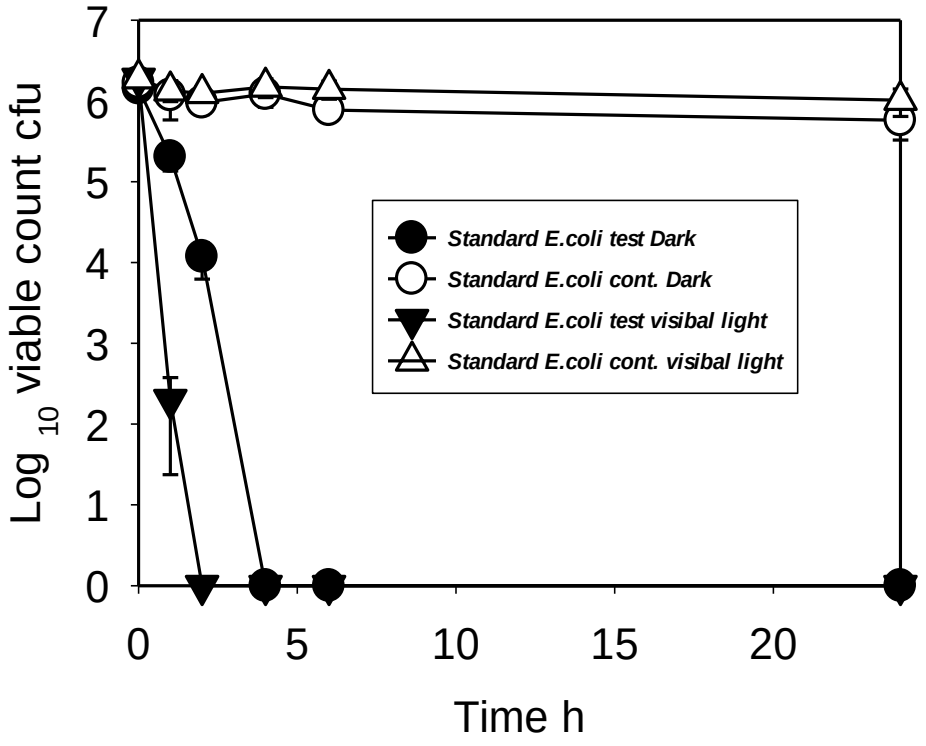

Figure 32 Killing of Standard *E. coli* (ATCC8739) on Cu/SiO_2 coated glass (test) and uncoated glass (control) under fluorescent light (UV 0.01 mW cm^{-2})

3.2.3.3 Retained bacterial cells from glass surfaces (coated and uncoated)

Reduction of bacterial numbers may have been due to binding of the organisms on the Cu/SiO_2 surface rather than by killing. The samples were therefore stained with crystal violet after re-suspension of the bacteria in TSB following incubation. The results showed that there were no bacteria detected under the microscope on both coated (test) and un-coated (control) samples.

3.2.3.4 Determination of MIC of copper in re-suspension medium

Although samples were processed as quickly as possible after incubation it is possible that Cu eluted from the coated surfaces continued to kill the bacteria. The MIC/MBC for Cu was determined in the re-suspension medium ISB. MIC determination for each strain was measured visually and by microtitre plate reader (O.D 570 nm). Microbial growth was considered as positive in the wells that showed any increase in turbidity. The last well in the dilution series that did not demonstrate any growth corresponds with the MIC of copper. The MIC after 4 h for Gram-positive (standard *S. aureus*) was 0.5 mM (0.125 mg/ml) which is greater than the MIC of Gram-negative (Standard *E. coli*) which was 0.25 mM (0.06 mg/ml) as shown in Table 5. This was confirmed after 24 h of incubation as shown in Figures 33, 34 and Table 5. There was no growth or the growth inhibited at the concentration of 0.25 mM for *S. aureus* after 24 h of incubation (MBC) however *E. coli* was highly turbid at this concentration after 24 h. These MIC values are higher than the concentrations shown to be eluted from the surface (20–70 µM) measured by inductively Coupled Mass Spectroscopy (Abohtera- personal communication) but there may be locally higher concentrations near to the surface of the coating. Alternatively, contact between the bacterium and the copper islands may allow diffusion of Cu^{2+}directly into the cell wall/membrane.

Table 8 Minimum inhibitory concentration of copper for standard *S. aureus* (ATCC6538) and standard *E. coli* (ATCC8739) after 4 h and 24 h

MIC after 4h			MIC after 24h	
mM copper	***S. aureus***	***E. coli***	***S. aureus***	***E. coli***
4	0.0867	0.0910	0.0424	0.0511
2	0.1180	0.1257	0.1079	0.1241
1	0.1463	0.1374	0.1656	0.1729
0.5	0.1474	0.1624	0.1338	0.1712
0.25	0.1106	0.1343	0.0851	0.5742
0.125	0.0916	0.0822	0.6230	0.9905
0.06	0.0870	0.0887	0.5515	1.3199
0.03	0.0813	0.0718	0.5699	1.4940
0.015	0.0762	0.0677	0.5176	1.6315
0.008	0.0745	0.0847	0.5254	1.4880
Control (+)	0.0683	0.0628	0.6403	1.1131
Control (-)	0.0438	0.0437	0.0379	0.0499

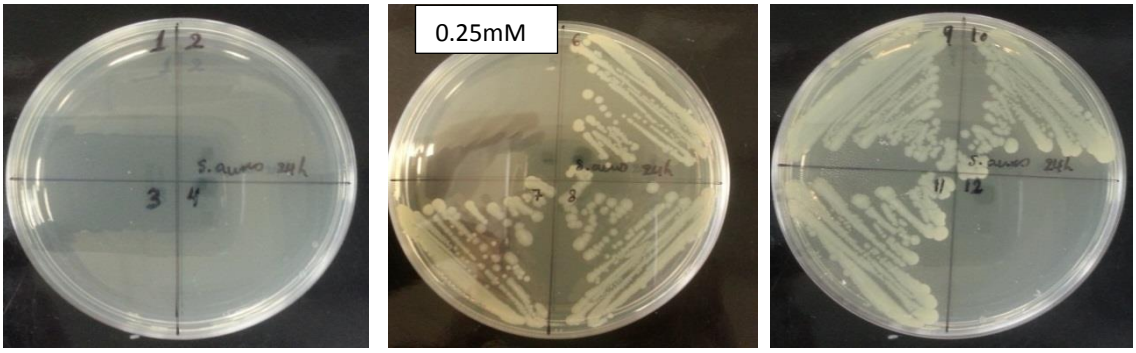

Figure 33 The 4 h MIC (of copper) of standard *S. aureus* (ATCC6538) after 24 h of incubation.

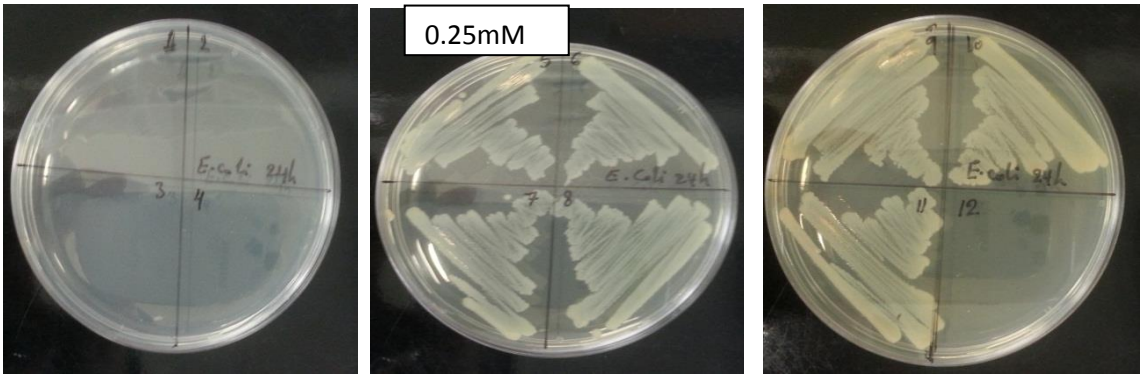

Figure 34 The 4 h MIC (of copper) of standard *E. coli* (ATCC8739) after 24 h of incubation.

3.2.3.5 Antimicrobial activity of coated ceramic tiles (Cu/SiO_2) at room temperature (25°C).

The activity of coated tiles was similar to coated glass with only 1 log reduction after 1h increased to >5 log reduction within 4 h. However on coated glass 2 log reductions was reached within 2 h and complete killing reached within 4 h (Figure 25). The activity of coated ceramic tiles on standard *E. coli* is shown in Figure 35.

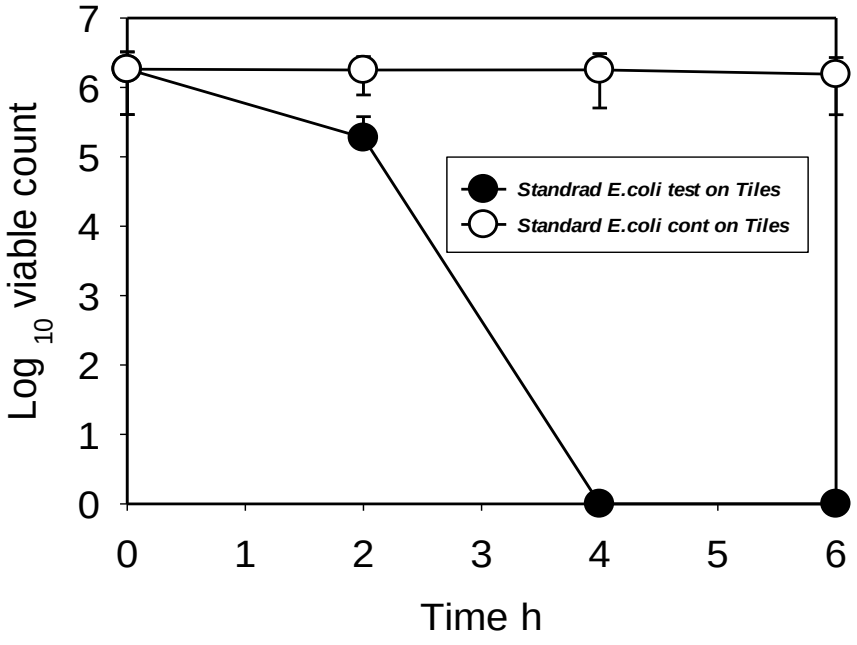

Figure 35 Killing of standard *E. coli* (ATCC8739) on Cu/SiO_2 coated tile (test) and uncoated tile (control) in the dark at room temperature (25°C).

3.2.4 The antimicrobial activity of CVD coated steel and painted steel

Steel and painted steel coated with different concentration of Cu, samples were named as 2, 2C, 5, 2b and 2b1. These had a silica base layer, a copper layer and a silica topcoat. Samples 2c and 5 had higher concentration of copper than 2, 2b and 2b1 and 5 had a thicker silica topcoat. These were tested for activity against bacteria using the modified BS ISO 22196:2009 & 2011 method as described in chapter 2. Two different controls were used plain steel/painted steel and silica. The activity of painted steel samples on *S. aureus* and *S. enterica Typhimurium* were similar and the highest reduction was seen on sample 5, with 4.5 log reduction for *S. aureus* ($P \geq 0.0001$) and 3 log for *S. enterica Typhimurium* ($P \geq 0.0001$) after 24 h as shown in Figures 39b and 40b. This compares to only 0.5 log reduction on 2b and 2b1 for both strains (Figures 39 a, and 40 a). *E. coli* was more sensitive than other strains tested, with ≥ 5 log reductions after 24 h on 2C, and 3 log on 2b and 2b1 as shown on Figures 38a and 38 b. On steel samples, the activity of *Salmonella* which is known to be responsible for food borne infections and able to survival on food surfaces for long period was more sensitive than the standard test strain of *S. aureus* with 3.5, 4 and 4.2 log reductions on 2b, 2C and 5 respectively compared to 3 logs on both 2C and 5 samples and only 1 log reduction on 2b for *S. aureus* after 24h as shown in Figures 36a and 36b. The activity against *S. enterica Typhimurium* is shown in Figure 37a and 37 b. The different log kills was due to different concentration of copper on the coated samples, and due to shortage in the steel samples *E. coli* was not tested. The samples with high concentrations of Cu showed the highest log killing (2C, 5).

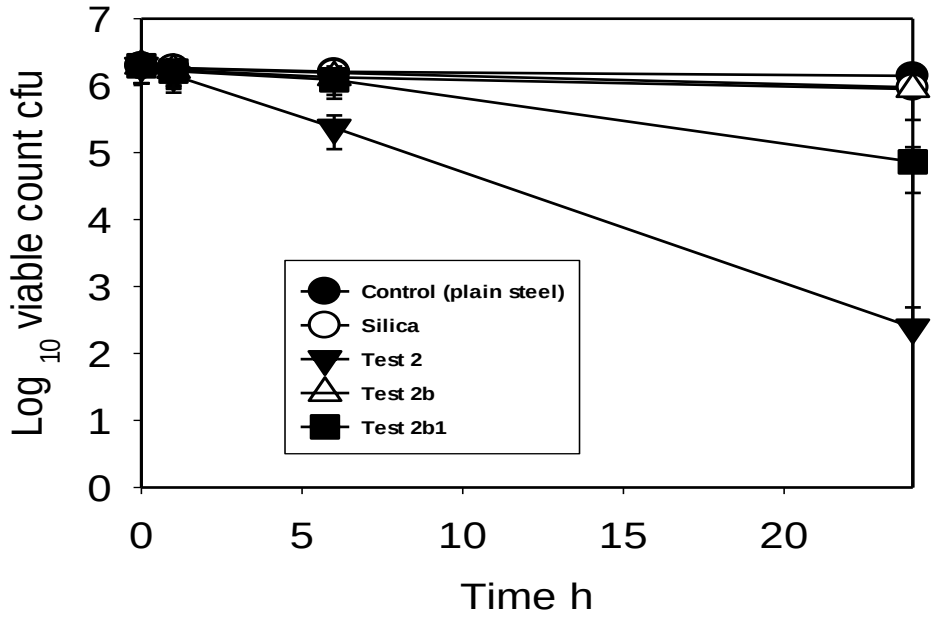

Figure 36 a Killing of standard *S. aureus* (ATCC 6538) on different samples of low Cu coated steel (2, 2b and 2b1-tests), silica and uncoated steel (control).

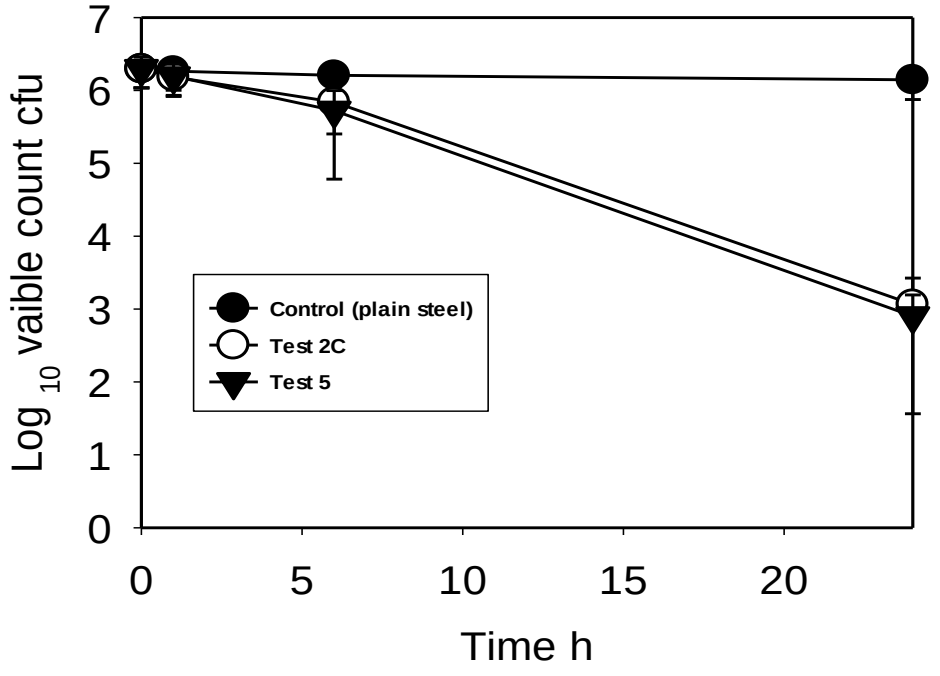

Figure 36 b Killing of standard *S. aureus* (ATCC 6538) on different samples of high Cu coated steel (2C, 5-tests) and uncoated steel (control).

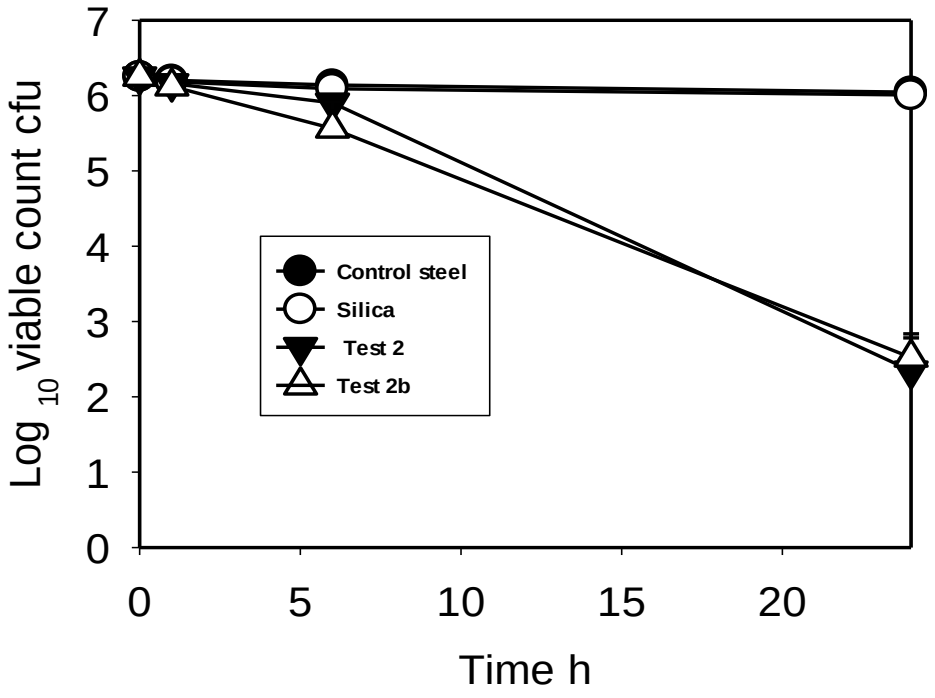

Figure 37a Killing of *S. enterica Typhimurium* on different samples of low Cu coated steel (2, 2b -tests), silica, and uncoated steel (control).

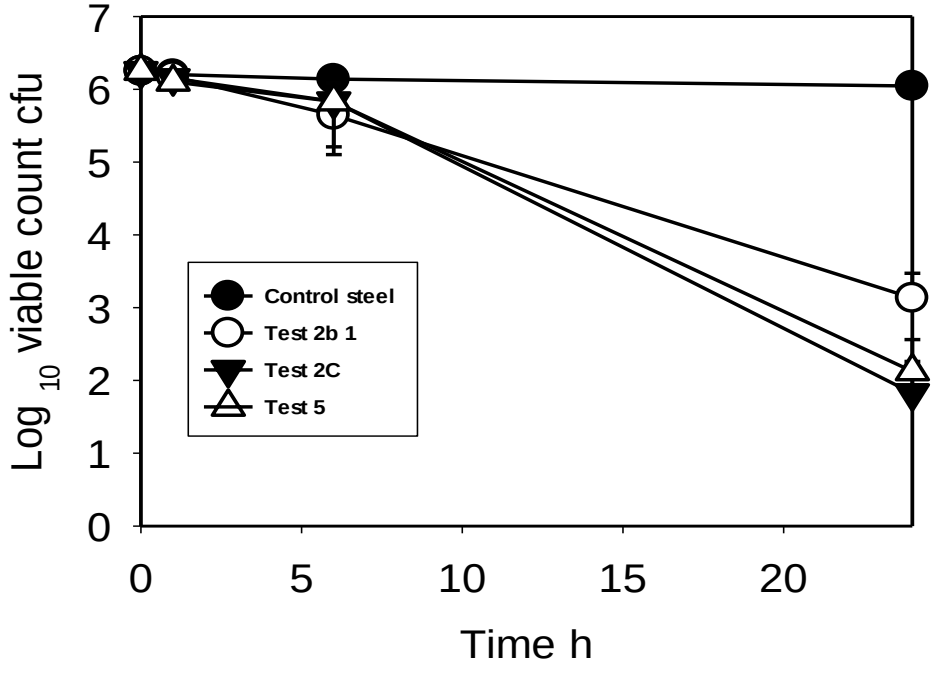

Figure 37b Killing of *S. enterica Typhimurium* on different samples of Cu coated steel (low Cu 2b1 and high Cu 2C, 5 tests) and uncoated steel (control).

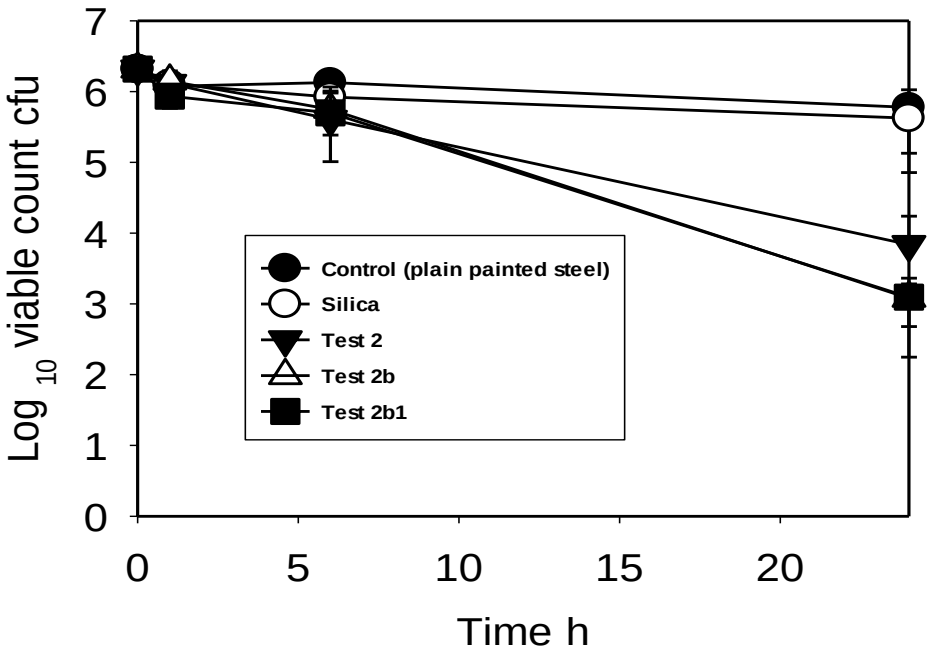

Figure 38a Killing of standard *E. coli* (ATCC8739) on different samples of low Cu coated painted steel (2, 2b and 2b1-tests), silica and uncoated painted steel (control).

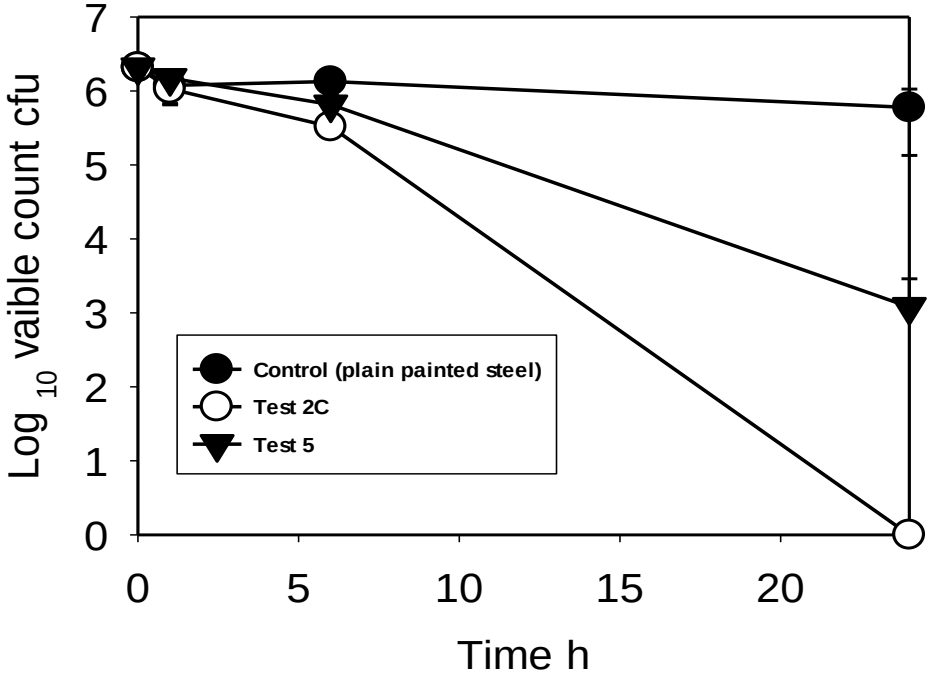

Figure 38b Killing of standard *E. coli* (ATCC 8739) on different samples of high Cu coated painted steel (2C, 5-tests) and uncoated painted steel (control).

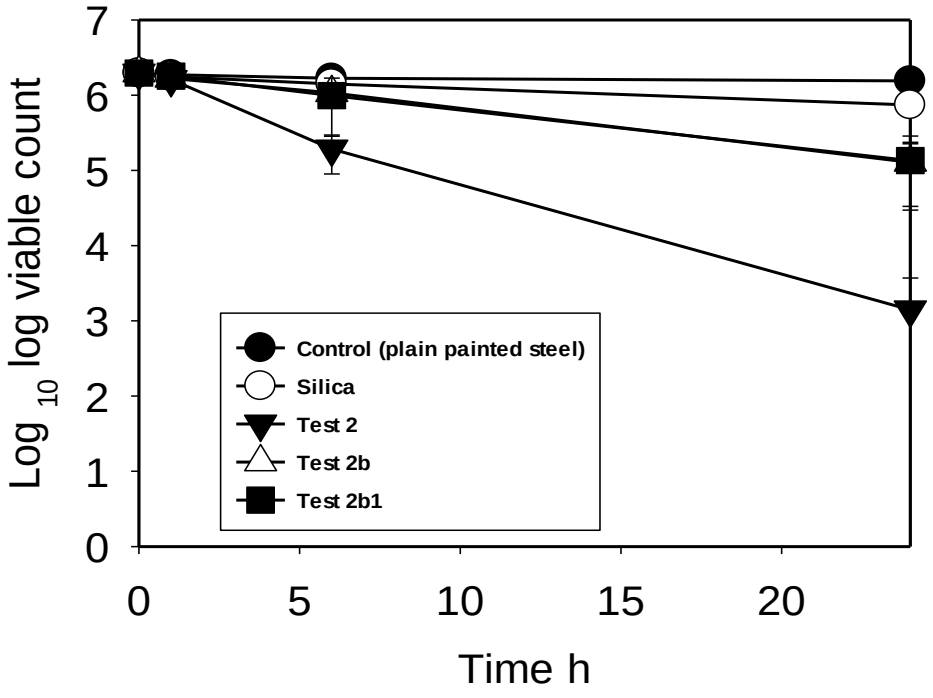

Figure 39a Killing of standard *S. aureus* (ATCC 6538) on different samples of low Cu coated painted steel (2, 2b and 2b1-tests), silica and uncoated painted steel (control).

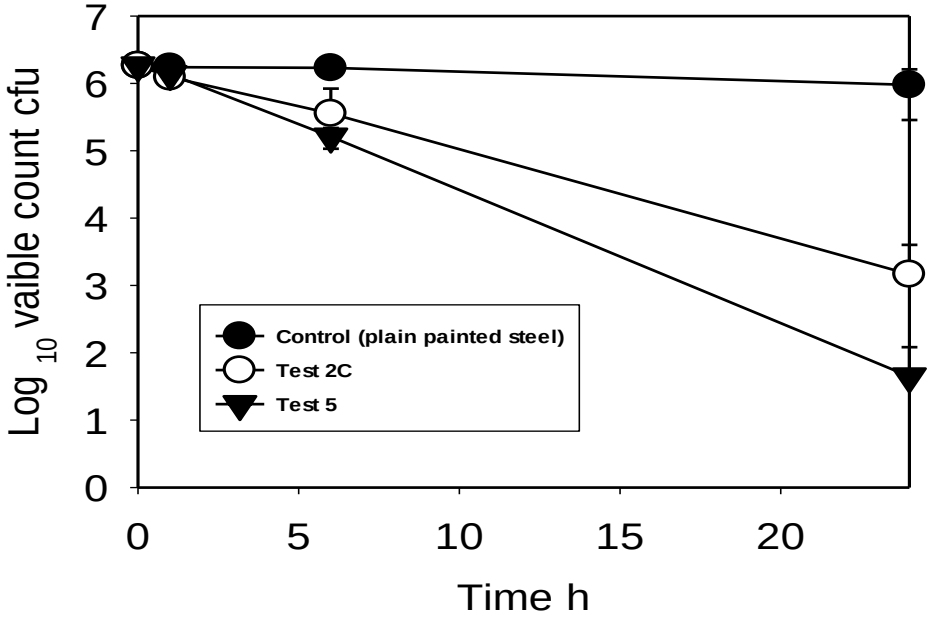

Figure 39b Killing of standard *S. aureus* (ATCC 6538) on different samples of high Cu coated painted steel (2C, 5-tests) and uncoated painted steel (control).

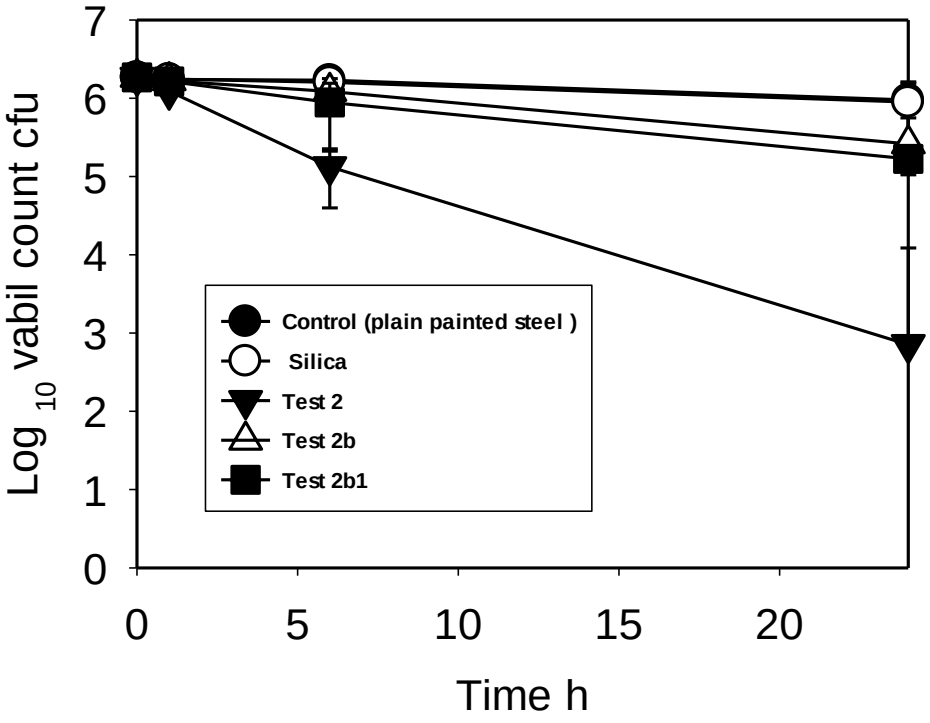

Figure 40a Killing of *S. enterica Typhimurium* on different samples of low Cu coated painted steel (2, 2b and 2b1-tests), silica and uncoated painted steel (control).

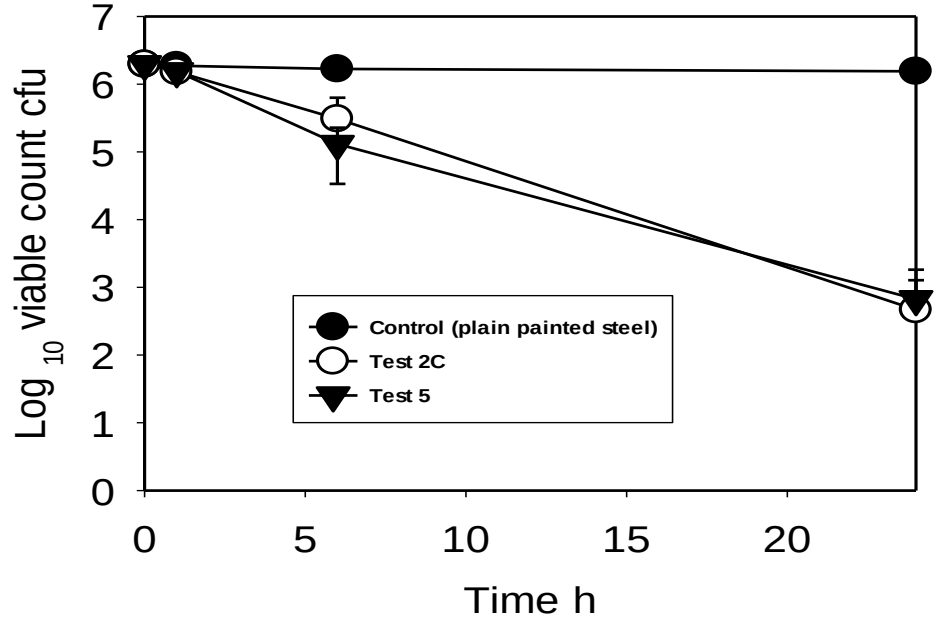

Figure 40b Killing of *S. enterica Typhimurium* on different samples of high Cu coated painted steel (2C, 5-tests), silica and uncoated painted steel (control).

3.3 Discussion

In this study a range of important pathogenic strains that cause HACIs were exposed to CVD glass coated surfaces (Ag/SiO_2, $CuO\text{-}TiO_2$, and Cu/SiO_2) and CVD coated stainless steel and painted stainless steel surfaces. Results show that the activity of CVD glass coated surfaces was greater than the activity of steel coated surfaces.

3.3.1 Antimicrobial activity of CVD (Salford University) coated surfaces

In general, all CVD coated substrates used in this study were more active against Gram-negative bacteria than the Gram-positive bacteria. The highest activity against hospital-related pathogens was seen in Cu/TiO_2 coated films followed by Cu/SiO_2 coated films.

Silver/Silica

The results show that the antimicrobial activity of Ag films was dependent on the amount of Ag in the film. Coated films with high amounts of Ag (0.25M) were more active than films with low amounts of Ag (0.05M). These films were also more active against Gram-negative than Gram-positive bacteria, with >5 log reduction on *K. pneumoniae* after 6 h and only 4 log killed after 24 h for *S. aureus* (standard test strain). These results were in line with a previous study conducted by the research team. *E. coli* and *S. aureus* were treated with three different concentrations of silver (0.05, 0.25 and 0.5 M). The highest log killing was seen on 0.5 M of silver and the lowest log killing was seen on 0.05 M of silver and this was constant for both types of bacteria tested. However, the hardness of the films was also affected (Cook *et al*., 2011, Varghese *et al*., 2013a). A similar finding was also obtained by Jia and others. They found that the contact time required to kill all viable cells of *E. coli* reduced from 6 h to 4 h when the amount of silver was increased (Jia *et al*., 2008). The increase in antimicrobial activity of silver surfaces that contain higher amounts of silver was due to

increased silver ions dissolved from the surfaces. Chen and others had investigated the relation between the silver dose and the amount of silver ions released and their antimicrobial activity. They suggested that the concentration of silver ions released increased from 35±16 ppb at a silver concentration of 5×10^{15} ions/cm^2 to 255±20 ppb when higher concentrations of silver were used (1000×10^{15} ions/cm^2), and their antimicrobial activity was consistent with both concentration of silver used and ions released. Thus, silver ions are crucial for antibacterial properties (Chen *et al*., 2013).

Numerous studies have previously reported that Gram-negative bacteria are more sensitive to silver than Gram-positive bacteria and they attributed it to differences in cell wall structure (Kim *et al*., 2007, Egger *et al*., 2009, Kim *et al*., 2011). It is well-known that Gram-negative bacteria contain an outer membrane on top of the peptidoglycan layer which is lacking in Gram-positive bacteria. The main function of the outer membrane is to act as a selective permeability barrier to penetrating nutrients to assist bacterial growth and to protect bacteria from harmful materials such as toxins, detergents and drugs (Amro *et al*., 2000). The effect of silver on the bacterial cells, *S. aureus* and *E. coli* cells were treated with 100 µg/ml of silver for 3 h and the morphological changes of bacterial cells were also observed. The cell surface of control cells (untreated bacteria) was intact and damage was not seen for either of the bacterial strains. However, in the cells treated with silver cell surfaces were changed due to increased cell membrane permeability and many fragments were formed through the damage of cell membranes. The cell fragments were possibly due to the products derived from the leakage of cytoplasmic contents in damaged cells. In fact, the effect was quicker in the case of Gram-negative bacteria than in the Gram-positive bacteria (Kim *et al*., 2011).

Silver cations are more toxic to bacteria than copper cations since silver cations bind more strongly to thiols than copper cations. Due to this property, silver surfaces may also be suitable to limit the

spread of bacteria in healthcare sectors. However, silver is not stable in Ag (II) oxidation form and should not catalyse a Fenton-Reaction (Mikolay *et al*., 2010). In this study, copper-containing materials were more efficient as antimicrobial materials than silver-containing materials (99.99% of *S. aureus* was killed on Cu/SiO_2 compared to 99.9 on Ag/SiO_2 after 24 h). In another study, Yoon *et al*., (2007) showed that both types of bacteria (Gram-positive and Gram -negative) were more sensitive to copper than silver. However, their results also showed that Gram-positive bacteria (*B. subtilis* -vegetative cells) were more sensitive to copper than *E. coli* (Gram–negative). They reasoned that the outer membranes of Gram-negative bacteria, which usually consist of tightly packed lipopolysaccharide (LPS) molecules, provided an effective resistance barrier against nanoparticles (Yoon *et al*., 2007). In a further study, researchers demonstrated that the biocidal effect of silver was higher than copper in both types of bacteria. Indeed, Gram-positive bacteria were more sensitive to silver than Gram-negative. They showed that to reach ≥ 3 log kill of bacterial cells 1-2 $\mu g/cm^2$ of silver was required, while in the case of copper the amount needed was almost ten times higher (10-15 $\mu g/cm^2$). Thus, the bactericidal activity of silver is not mainly dependent on the structure of the bacterial membrane (Esteban-Tejeda *et al*., 2012). However, these studies differed from the present study in that, in this study bulk copper/silver was co-deposited with silica which may lead to the delay in the release of copper/silver ions from the surfaces and thus cause killing delay compared to one layer coated nanoparticles (copper/silver) surfaces which known to release higher amounts of copper/silver ions.

The antibacterial efficacy of silver-containing polymers is dependant on the release of silver ions which produced due to the interaction between silver and water (Kumar and Münstedt, 2005). In spite of the fact that the bactericidal effects of silver ions have been intensively studied for more than 60 years (Li *et al*., 2010), the exact mechanism of inhibitory action of silver on microorganisms is still not fully understood (Sondi and Salopek-Sondi, 2004, Cho *et al*., 2005, Egger *et al*., 2009, Li *et*

al., 2010). Interestingly, it had been reported that the action of silver ions and silver nanoparticles are broadly similar (Pal *et al*., 2007b). Some previous reports in the literature showed that the ionic attraction force between negatively charged bacterial cells and positively charged silver surfaces is crucial for the activity of silver as bactericidal materials, as surface charge may influence nanoparticle interactions with living cells and thus their toxicity (Hamouda and Baker, 2000, Stoimenov *et al*., 2002, Pal *et al*., 2007b). Moreover, the effect of silver on *S. aureus* and *E. coli* grown with two different stabilizers (sodium dodecylsulfate -SDS and poly *N*-vinyl-2-pyrrolidone-PVP) were investigated. Results showed higher antimicrobial activity on silver with PVP compared to silver with SDS. There were two possibilities for their finding. One is that the negative charge of the SDS surface interferes with absorption of bacteria on the silver surface. The other is that dissolved or remaining silver ions interact directly with the negative charge of the SDS, and this reaction (electrostatic) may prevent the interaction between Ag ions and bacterial surface (Cho *et al*., 2005). On the other hand, silver particles used in some studies were negatively charged and showed antimicrobial activity against bacteria such as *E. coli* (Sondi and Salopek-Sondi, 2004). However, since those studies included both positively charged Ag ions and negatively charged of Ag, it is insufficient to explain the antimicrobial mechanism of positively charged Ag surfaces. Therefore, another possible mechanism should be addressed (Kim *et al*., 2007).

The inhibitory action of silver is also based on the release of silver ions Ag^{+} which may accumulate in the bacterial plasma membrane, causing an increase in permeability and cell death and penetrating bacterial cells (Egger *et al*., 2009). In a study, SEM microscopy was used to evaluate the effect of silver on the surface morphology of *E. coli* grown in a Luria-Bertani medium (LB). Results showed that the treated bacterial cells were significantly changed and showed major damage, which was characterized by the formation of pits in their cell walls; thus indicating that silver ions had penetrated into bacterial cells and interacted with membrane elements and caused damage to the

integrity of the cell walls (Sondi and Salopek-Sondi, 2004). Cho and his team showed that the cell walls of both types of bacterial strains (*E. coli* and *S. aureus*) were affected by silver (Cho *et al*., 2005). In contrast, another study showed greater biocidal activity of silver against *E. coli* compared to *S. aureus* and inferred it to be due to the difference in cell wall structure between Gram-positive and Gram-negative bacteria (Kim *et al*., 2007). However, these conclusions are not completely acceptable because there is insufficient evidence to support them, since most research on the bactericidal effects of silver has been used against one, or a very limited number, of microbial strains (Ruparelia *et al*., 2008). Another possible mechanism of the biocidal effects of silver suggested is that the biocidal activity may be related to membrane damage due to free radicals (ROS) that are released from the silver surface and the uncontrolled generation of free radicals can interact with membrane lipids leading to a disruption of membrane permeability (Kim *et al*., 2007). In the same study, N-acetylcysteine (NAC) was used as an antioxidant to investigate the relationship between the production of free-radicals (ROS) and the antimicrobial activity of silver. Results showed that similar antimicrobial activity was seen between Ag nanoparticles and silver nitrate against *E. coli*. However, the antimicrobial activity of Ag nanoparticles and silver nitrate was influenced by NAC. It is suggested that free ions may be derived from the surface of Ag nanoparticles that are responsible for the antimicrobial activity (Kim *et al*., 2007). Moreover, high levels of ROS can also cause damage to the proteins, DNA and intracellular systems such as the respiratory system (Kim *et al*., 2011). It has been reported that the replication of DNA molecules can be effectively conducted only when DNA molecules are in a relaxed state, and in their condensed form DNA molecules lose their replication abilities (Feng *et al*., 2000). Thus, when silver ions penetrate into the bacterial cell, the bacteria clustered to protect the DNA from the silver ions and thus the multiplication of the cell well block. In order to confirm this hypothesis, the bacteria treated previously with silver were inoculated into a fresh liquid LB medium; however, no cell growth or cell multiplication was seen. This can be

explained by the fact that DNA molecules lose their replication abilities due to metal effects (Feng *et al*., 2000).

Copper/Silica and Copper/Titanium

The copper coated surfaces (Cu/SiO_2 and Cu/TiO_2) reduced the survival of all organisms tested by at least three logs, equivalent to a 99.9 % kill after 24 h. The greatest reduction on TiO_2 based surfaces was seen under UVA light, followed by fluorescent light and then in the dark. Dark activity was probably due to the release of Cu ions from the surface. It has been described that the copper species photo-deposited on TiO_2 particles on the TiO_2 film present as a mixture of CuO and Cu_2O (copper species), and if these species exposure to the air in the dark, dissolved and oxidization will occur (Sunada *et al*., 2003). Activity in light was probably due to the release of copper ions together with the production of reactive oxygen species by the CuO, which was further enhanced by ROS production by the TiO_2 with UVA illumination. $ESBL^+$*E. coli* were more resistant than the standard test *E. coli* but were killed after 6 h on copper titanium and after 24 h on copper silica. *K. pneumoniae* is well-known not only to cause serious hospital infections but also as a source of antibiotic resistance genes including extended spectrum B-lactamases. However, it was as sensitive to being killed by copper as a test strain on both types of surfaces (Cu/SiO_2 and Cu/TiO_2). Recently isolated strains of MRSA, which are known to be able to survival on a range of surfaces and objects for prolonged periods were also killed on copper surfaces.

Copper/Titanium

The results of this study demonstrate that only 1-2 log reduction was seen in the first 2 h; however, the killing reduction increased to higher levels after 4-6 h in all bacteria tested. This may indicate that the killing of bacteria on copper /titanium films irradiated by light is achieved through a two-

step process, a slow one followed by a strong, fast step. In the first slow step, the rate of killing is related to the concentration of a single substance increased to the first power, which could be the ROS produced by TiO_2. In the first-order reaction, the initial concentration of one substrate (reactant) is much larger than the second substrate (reactant). The concentration of the first reactant will remain almost stable. Therefore, the reaction rate is based on the second reactant, which in this study is the ROS generated by both copper and titanium, and copper ions, and the photocatalytic reaction assists the copper ions to penetrate into the cell. The two step killing was also demonstrated by another study (Tsuang *et al*., 2008). On the other hand, Horie and others suggested the opposite effect. They showed that the photocatalytic killing process of TiO_2 surfaces occurs through a series-event model. They suggested that the photocatalytic cell killing was dependent on a second-order reaction between cells and oxidative radicals, and the death of a cell is induced by *n* time's reactions on the basis of a series-event model (Horie *et al*., 1996). Suspension medium was reported as a factor that may have an effect on surface activity. It was reported that saline (sodium chloride), which was also used in the present study, enhanced the rate of the photocatalytic killing of bacteria on titanium coated surfaces. However, the opposite effect was seen when phosphate buffer saline (PBS) was used. This may occur by phosphate ions inhibiting the contact between surface particles and bacteria (Ditta *et al*., 2008). Indeed, the photocatalytic killing activity in the saline medium was also higher than that in the distilled water. Furthermore, if oxidisable material present in the re-suspension medium also competes with the bacteria for the generation of reactive oxygen species, even though the use of distilled water does stress the bacterial cells, it also does eliminate any variation in interference effects from ions and organic matter which is usually different on different surface (Ditta *et al*., 2008). This could be explained by two facts. Firstly, it is possible that the sodium chloride is increasing the adsorption of bacteria onto the photocatalyst, which leads to increased bactericidal activity of the surface. The other possibility is that Cl^- ions are affecting the permeability

of the bacterial cells, or that the photocatalyst is oxidising the Cl^- ions which leads to the formation of hypochlorite which has disinfectant properties (Cushnie *et al*., 2009).

In line with these results, a recent study suggested that surfaces that contain a single layer of TiO_2 had no effect on bacterial growth in the dark, but when mixed with copper the *E. coli* was killed within 120 min, and under UV light the *E. coli* was killed within 30 min. This indicates that the killing activity of TiO_2/Cu in the dark was due to the release of copper ions, and the killing under light was due to reactive oxygen species produced by both copper and titanium (Baghriche *et al*., 2013). TiO_2/Cu binds tightly to the negative groups of bacteria cell walls, and this interaction between bacteria cell wall compounds and the TiO_2/Cu film leads to damage of the cell wall envelope. This damage seems to be more important compared to the damage caused by Cu or TiO_2 films separately. Moreover, light irradiation activates charge transfer directly from the TiO_2 valence band to the Cu deposited on the TiO_2 surface, which results in multi-electron oxygen reduction acceleration, and both processes increase under a higher light dose. Therefore, an increase in light intensity leads to a shorter time to the killing of bacteria (Baghriche *et al*., 2013). This result is consistent with the previous study, which showed that the concentration of light intensity affects the toxicity of the TiO_2 surface (Horie *et al*., 1996). In fact, this was also confirmed recently by Foster and others. They showed that the fastest killing of *E. coli* was seen on TiO_2 irradiated with a high dose of UVA light (Foster *et al*., 2010).

TiO_2 photocatalysis can increase oxidation of membrane lipids and cause damage of cell respiration. The oxidation of membrane lipids caused by TiO_2 irradiated by UV light was first reported in 1999 by Maness *et al*. They demonstrated that the initial phase of lipid peroxidation started at an exponential phase, and this was based on the formation of malondialdehyde (MDA) which was used as an index of lipid peroxidation. The superoxide ions may react with the intermediate hydroperoxide

to start new radical chain reactions, assuming that the molecule can penetrate the cell membrane once its permeability is damaged. The series of autoxidation reactions will lead to the destruction of the membrane lipids, which means the destruction of the cell membrane itself. Therefore, since all life forms have a cell membrane made up of an array of lipids with different structures depending on their functions in the cells, the suggested killing mechanism is relevant to all cell types (Maness *et al*., 1999).

The results of the present study also showed that Gram-negative bacteria were more sensitive to killing by Cu/TiO_2 irradiated by light than Gram-positive bacteria (except VRE which was as sensitive as the Gram-negative *K. pneumoniae*), which appeared to be more resistant to the light-TiO_2 induced damage. This may be due to differences in the cell wall, Gram-positive bacteria have a much thicker peptidoglycan layer than Gram–negative bacteria (Dunnill *et al*., 2009). This may explain the longer killing time for *S. aureus* compared with the Gram-negative *E. coli*. . Therefore, a longer oxidation time or more photocatalysts are required for the destruction of Gram-negative bacteria. However, Gram-negative bacteria responded better to photocatalytic inactivation, indicating that cell wall destruction is probably not required for inactivation. Accordingly, the authors suggested that no significant cell wall destruction by photocatalytic oxidation could be observed. They hypothesised that Gram-positive and Gram-negative cells may modify different photocatalytic inactivation mechanisms (Liu and Yang, 2003). This hypothesis is in good agreement with other reports in literature reviews (Pal *et al*., 2007a). In contrast, some studies have shown the opposite, where Gram-positive species were more readily killed than Gram-negative species. Saito and others showed that Gram-positive *Streptococcus* strains were killed within 90 min under TiO_2 photocatalytic surfaces (Saito *et al*., 1992). In another study investigating bacteria cell killing using photosensitizer dyes and visible light, the sensitivity of Gram-positive *S. aureus* was found to be greater than Gram-negative *E. coli* (Demidova and Hamblin, 2005). Another possible mechanism of

photocatalytical inactivation is the leakage of intracellular substances such as potassium ions (K^+). The loss of potassium ions in bacteria causes loss of cell viability. Therefore, the leakage of K^+ has been used as indicator for membrane permeability changes. In bacterial cells that were exposed to TiO_2 and light irradiation, all K^+ had leaked from the cells in a short time parallel to the loss of cell viability. However, the addition of potassium ions could not retain cell viability; therefore the leakage of K^+ cannot be a single effect that causes bacterial death (Saito *et al*., 1992). It is well-known that TiO_2 is able to generate ROS when irradiated with light and these free radicals can react with the nucleic acids of bacterial cells. So it is possible that reactive radicals are penetrating to the cell through the peptidoglycan layer of the Gram-positive bacteria without causing any fatal damage, while complete damage might occur in the Gram-negative bacteria (Pal *et al*., 2007a).

The effect of fluorescent light on antimicrobial activity of Cu/TiO_2

It is well documented that TiO_2 is activated only with UVA light (320-400 nm) (Pal *et al*., 2007a). However, in the present study, the activity of Cu/TiO_2 coated films continues even under a florescent light which can emit a very small fraction of UVA light. In this case the activity of Cu/TiO_2 seen under visible light may be due to the photoactivity of CuO. CuO is a semiconductor that is able to generate a photoreaction under light of a longer wavelength than TiO_2 i.e. visible light due to its small band gap (1.7 eV; Baghriche *et al*., 2012) . Therefore, copper enhances the photocatalytic activity of TiO_2 by two different process - induced interfacial charge transfer from vb electrons of TiO_2 to Cu at the junctions between CuO and TiO_2 ,and by the enhanced production of ROS via a Fenton-type reaction as described in Chapter One (Sato and Taya, 2006). This was in good agreement with previous studies. Sunada and others showed that the survival rate of *E. coli* on Cu/TiO_2 films under visible light (1 $\mu W/cm^{2,}$ the UV intensity of indoor lighting) was almost the

same as that on film stored in the dark, which may due to the release of copper ions. Therefore the bactericidal activity of the TiO_2 photocatalytic reaction in the dark and under visible light was concealed by the activity of the copper ions (Sunada *et al*., 2003).

Even though that the activity of Cu/TiO_2 films under UVA light is higher than the activity of the Cu/TiO_2 films under visible light; the film would greatly enhance their usefulness in infection control.

Copper /silica

In this study, some pathogenic strains, such as *K. pneumoniae*, *A. baumanii* and *S. maltophilia*, which are known to survive for a prolonged period of time on inert surfaces, were as sensitive to copper silica as standard test strain (*E. coli* ATCC8739) and were killed within 4 h. However, others were more resistant and were only killed within 24 h, such as *Salmonella* and ESBL producing *E. coli*. The survival time of bacteria on surfaces has been shown to vary in different studies and under different conditions (Kramer *et al*., 2006). It is controlled by many factors such as type of surfaces, medium (Noyce *et al*., 2006b), inoculation method (Santo *et al*., 2008), inoculum size (Warnes *et al*., 2010), temperature and humidity (Souli *et al*., 2012). The effect of temperature on copper activity will be discussed the following chapter.

The effect of different media on copper activity

Molteni and others showed that there was a correlation between types of media, the amount of copper released from copper coupons and the killing rate. Wild type and mutant (*CopB) E. hirae* were grown in four different media: Tris-Cl, water, M17 medium and phosphate. Results showed that the highest rate of killing was seen on the Tris buffer for both types of *E. hirae* (mutant and wild type); complete killing occurred after 10 and 12 min respectively. Indeed, the highest amount of

copper accumulated in the cells was also seen in Tris which was 42 mM. In the M17 medium, which accumulated 14 mM of copper, complete killing of the wild type took 90 min. In the water, which dissolved only 55 µM of copper, complete killing of the wild type took 6 h; similar killing rates were seen in the phosphate buffer, which dissolved 57 µM of copper. Tris is known to form strong copper complexes. On the other hand, the phosphate copper complexes that were also shown to occur are comparatively weak. Thus, the fact that no strong killing activity was seen on phosphate compared to Tris suggested that the more rapid killing in the Tris buffer is a specific property of this buffer substance (Molteni *et al*., 2010). Similar to that found in a recent work, the survival rates of *E. coli* and *E. faecium* cells were dependent on the corrosion inhibitor's (Benzotriazole, BTA) concentration in the solution in which copper surfaces were treated. *E. coli* suspended in 0.8% NaCl and placed as a drop on electroplated copper surfaces were killed within 60 min of exposure to copper surfaces, but there was no effect after 60 min of exposure to BTA-coated electroplated copper. However, a different effect was obtained on *E. faecium*; CFU were decreased by 3 log after 60 min on the BTA-coated surface but there was no CFU detected after 45 min on the copper surfaces. These results suggested that exposure of copper surfaces to 0.016 M of BTA solution resulted in a decreased level of copper release and rate of bacteria killing (Elguindi *et al*., 2011). However, these mechanisms are affected only on moist copper surface conditions and offered no protection under dry conditions, and most likely allowed a very rapid and massive influx of copper ions into cells, leading to cell death (Elguindi *et al*., 2011, Hans *et al*., 2013).

The effect of inoculation method wet verses dry

Previously, a series of studies were conducted to investigate the effect of copper surfaces on the survival time of different bacteria using wet inocula, such as *E. coli* O157 (Wilks *et al*., 2005), *P. aeruginosa* (Mehtar *et al*., 2008, Gould *et al*., 2009), *A. baumanii* (Mehtar *et al*., 2008), *E. faecalis*,

S. aureus and *K. pneumoniae* (Mehtar *et al*., 2008, Varghese *et al*., 2013b). However, the wet inoculum (a few microliters of a bacterial suspension are applied to the copper surfaces as a droplet) does not correspond to contamination of copper touch surfaces with bacteria in health care environments, which are usually dry. Santo and others had used dry inoculum (a bacterial suspension is spread on the copper surfaces, which allows the water to evaporate in seconds) to investigate the effect of copper against bacteria. A complete killing of *E. coli* was achieved within minutes on dry surfaces, while the in aqueous solution survival time of *E. coli* was longer. The killing in both methods was related to copper ions being released from the copper surfaces and accumulating in cells. However, in the case of dry surfaces the amount of copper ions accumulated by cells was higher than that of the wet surfaces (Santo *et al*., 2008, Santo *et al*., 2011). Moreover, bacteria on dry surfaces do not have time to develop biofilms and the stress and survival conditions are different from those of aqueous surfaces. However, when bacteria were exposed to different metallic surfaces and under the same dry condition stress, the bacterial growth was not inhibited. *E. coli* O157 was able to survival in dried deposits on stainless steel for periods of 6 h. In contrast, complete killing of 10^7 cells was achieved after 75 min on copper alloys (Noyce *et al*., 2006b). Therefore, dehydration stress is not the primary mechanism of quick killing in the dry method (Faúndez *et al*., 2004, Noyce *et al*., 2006b, Santo *et al*., 2008). One drawback of the dry method technique is that the results were not reproducible as with the wet method, since the amount of bacteria left on the surface was not standardized (Souli *et al*., 2012). In the present study, the wet technique was used, which was the most common method used in related published literature, in order to have reproducible results and to be able to compare the results with previous findings.

Even though the dry method is more effective than the wet one, both techniques were controlled by the rate of release of copper ions from the metal surface. And a slower release rate reduces the contact killing (Molteni *et al*., 2010, Elguindi *et al*., 2011, Mathews *et al*., 2013). Copper ions are

able to kill bacteria by damaging their cell wall and cell membrane because Cu ions can extract the electrons from bacteria, causing damage to their cytoplasm and oxidizing their cell nucleus. It has been reported that the types of medium also affect copper toxicity, since some organic and inorganic ions of the medium react chemically with metal ions and reduce copper toxicity (Zevenhuizen *et al*., 1979). These findings indicate that the toxicity of heavy metals are not related to the total metal concentration of the medium but are dependent on the particular chemical state in which they exist (Zevenhuizen *et al*., 1979). In a recent study, the effect of copper on *E. coli* grown with EDTA complex was investigated. They found that there was no effect of copper bearing antimicrobial stainless steel on the survival of bacteria cells when EDTA was used. Their results indicate that some copper ions should exist to cause bacteria death on copper bearing antimicrobial stainless steel. On the other hand, there was no difference in the number of bacteria colonies on plain stainless steel, with and without EDTA. This can be explained by the fact that a certain concentration of EDTA in the bacteria solution interacts with Cu ions that dissolved from the copper surface and thus reduced its toxicity. Indeed, the morphology of cell walls and cell membranes of *E. coli* was also affected by copper ions, and cellular contents in the cytoplasm were leaked (Nan *et al*., 2008), and this had also been reported in many studies (Ren *et al*., 2009, Rodriguez-Llamazares *et al*., 2012). Another comparison of copper toxicity between copper ions and copper complex was carried out on *E. coli*. Results showed that there was a 99% reduction in cell count when cells were treated with 200 µM of Cu^{2+} ions whereas cells treated with different copper complexes (Cu-EDTA, Cu-NTA, and Cu-IDA) remained almost unaffected. This indicates that Cu^{2+} ions in a free state can show significant toxicity to *E. coli* while copper ions in complex forms were not toxic. The high toxicity of free copper ions was related to the saline medium used, so that copper could exist in a free state and cells could not regulate the concentration of cellular copper. Low toxicity of copper complexes indicates that there

is no accumulation of Cu inside bacteria cells. This means that the copper complexes are stable, non-labile and inert towards their microbial toxicity (Selvaraj *et al*., 2009).

The effect of inoculum size on copper activity

Inoculum size is another factor that may affect the toxicity of copper. To investigate the effect of inoculum size on time required for total kill on copper coupons, the coupons were tested with inocula of different MRSA strains ranging from 10^2 to 10^7 CFU. Results suggested that as the inoculum size was reduced the time required for complete killing was also reduced. However, the same killing time (90 min) was required for both sizes of inoculum 10^6 and 10^7 CFU, and 75 min was required for 10^5 and 10^4. For the lowest inoculum size of 10^3 and 10^2 CFU, 45 and 30 min were needed respectively, for complete killing to occur (Noyce *et al*., 2006a). Similar results were found for *E. faecalis*. There were a low number of viable cells remaining after 2.5 h when 10^7 of *E. faecalis* were inoculated on copper alloys. However, reducing the inoculum to 10^5 resulted that no viable cells were detectable within 60 min. In fact, on pure copper all cells were killed within 20 min at an inoculum concentration of 1000 cells/cm^2 (Warnes *et al*., 2010). Moreover, reducing the number of CFU of *E. coli* O157 exposed to dry copper alloys from 10^7 to 10^3 resulted in a complete kill within 20 min (Noyce *et al*., 2006b). In contrast, in the present study there was no significant effect of inoculum size on killing time. A $\geq$ 5 log a reduction was achieved after 4 h in both concentrations 10^5 and 10^6 and after 2 h when 10^3 was used. In fact, the actual killing time in this study may be quicker than the one indicated, but a short time (every 60 min) was not tested. However, the other studies used dry inocula rather than wet inocula, and a different bacterium (*E. coli*) was used in the present study.

Another possible mechanism of killing by copper is the different electricity charge between copper and bacteria surfaces. The overall charge of the *E. coli* cell wall is negative due to the large amount of carboxylic external groups located in the cell wall. This opposite charge on the bacteria and the

Cu positive ions may induce the binding of the Cu to the bacterial surface (Castro *et al*., 2010).

Copper resistance mechanisms

Bacteria respond differently to abnormally high metal concentrations, similar to all living organisms, and a number of bacteria have been reported as copper-tolerant organisms able to accumulate the metal ions in their cells and thus preventing their entry into the cells (Hookoom and Puchooa, 2013). To survive under metal-stressed conditions, bacteria have developed a variety of resistance mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the formation and isolation of heavy metals in complexes inside the cell, reduction of a metal to a less toxic state, and direct efflux of a metal out of the cell through specific genes (Teitzel and Parsek, 2003), and some of these genes that are involved in copper resistance and homeostasis can affect the survival ability of bacteria on copper surfaces. It has been found that *E. coli* possesses three major systems for protection against copper ion toxicity*; CueO, Cus* and *CopA*. *CueO* is an oxidase that oxidizes periplasmic copper ions to a copper complex to prevent its entry to the cytoplasm. The *CopA* pumps copper from the cytoplasm into the periplasm. And the *Cus* system pumps copper from the periplasm back to the environment (Santo *et al*., 2008, Macomber and Imlay, 2009, Elguindi *et al*., 2011). The killing of mutant *E. coli* (deleted in *CueO, Cus* and *CopA*) was faster than the wild type strain on copper surfaces (Santo *et al*., 2008). Similarly, the *E. hirae* mutant lacked the *copB* gene, which is responsible for the copper export pump, and was killed after 75 min of exposure to copper, while complete killing of the wild type strain took 90 min. Hence, these genes are related to copper ion detoxification; so these results suggest that ionic copper is the primary fatal agent of copper toxicity (Santo *et al*., 2008). In contrast, copper had no significant effect on the survival of *E. hirae* mutant *CopA* (encoding the copper import ATPase) compared to the wild type strain. This indicates that the copper homeostatic mechanism provides the wild type strain with a protection against killing, and

only mutants that were unable to resist copper were killed more rapidly than the wild type strain on copper surfaces (Molteni *et al*., 2010). Moreover, an experiment was conducted on single gene mutants to evaluate which of the three copper resistance systems (*CueO, Cus* and *CopA*) is an important factor for the bacteria to survival on copper surfaces. Additionally, mutants lacking periplasmic (*CueO, Cus*) or cytoplasmic (*CopA*) copper resistance systems were also studied to investigate which mutant is more susceptible to copper. Results showed that single or double mutants were not as sensitive to copper as triple mutants, and the three copper systems are necessary for the bacteria to survive under copper stress. In fact, the *Cus* mutant showed a survival rate similar to that of the wild type strain (Santo *et al*., 2008), and the reason for this action could be contributed to the fact that the *Cus* system is responsible for periplasmic Cu ion detoxification, mainly under anaerobic conditions (Outten *et al*., 2001). Furthermore, under anaerobic conditions, the toxicity of copper to *E. coli* was due to the shift in total copper from the Cu^{+2} to the Cu^{+1} oxidation state. This may occur by biological reduction mechanisms such as cell surface reductase, small molecule reductants or other components of the electron transport machinery. The fact that Cu^{+1} is more toxic than Cu^{+2} suggests that the role of *CueO* during aerobic growth may be to convert periplasmic copper from a highly toxic form (Cu^{+1}) to a less toxic form, namely Cu^{+2}. Therefore, the absence of oxygen is a strong factor that causes the loss of *CueO* function and thus its toxicity (Outten *et al*., 2001). Evidently copper resistance systems do not protect bacteria from contact killing, but they offer a prolonged survival (Grass *et al.*, 2011). This may explain the different effects of copper, not only between Gram-negative and Gram–positive bacteria, but also between bacterial strains from the same genes, as shown in this study. The relative susceptibility of bacteria strains tested in this study to copper is shown in Figure 41a and b. However, these mechanisms are only affected on moist copper surface conditions and offered no protection under dry conditions, and most likely allowed

very rapid and massive pumping of copper ions into the cells, leading to cell death(Elguindi *et al*., 2011).

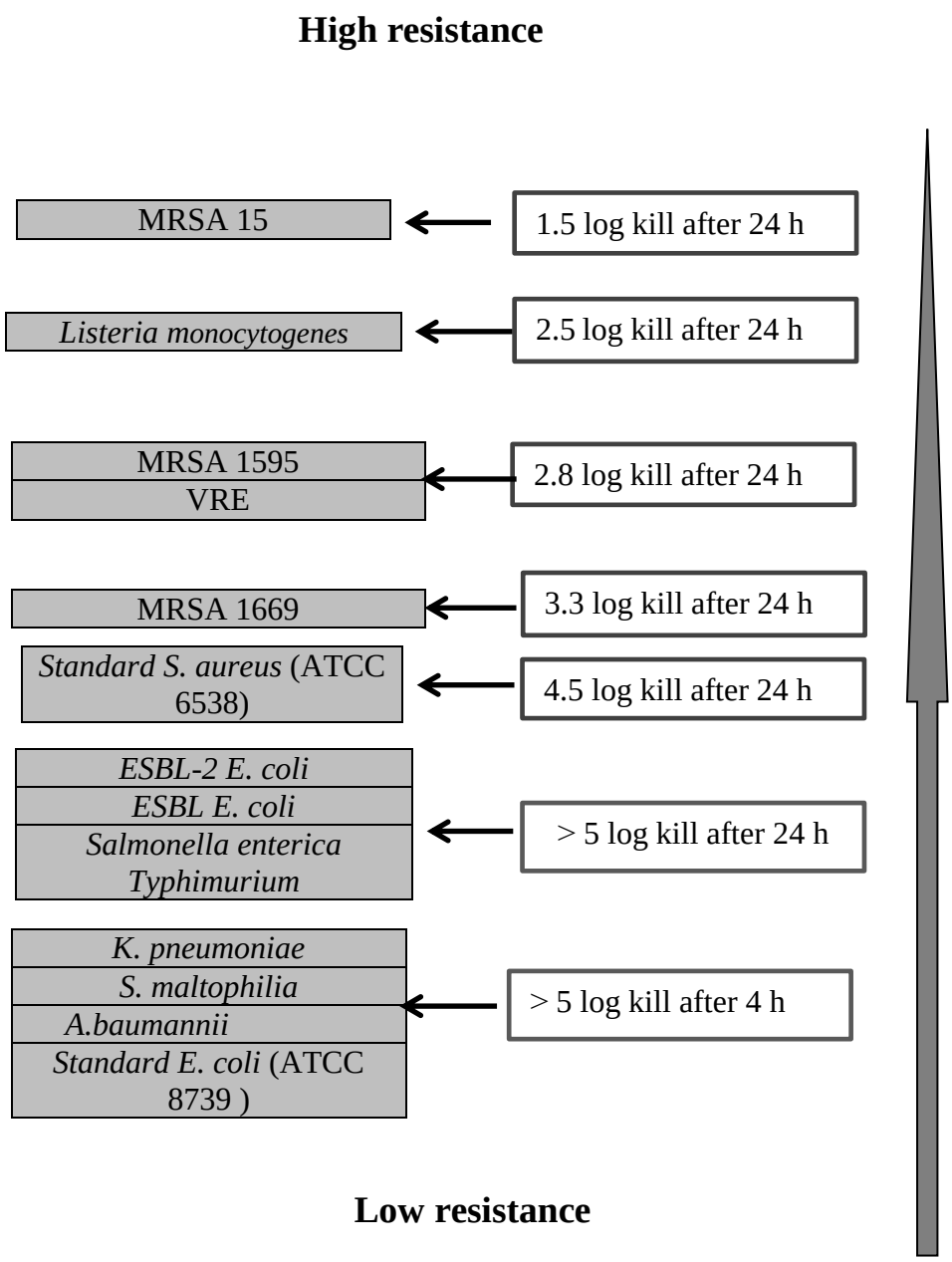

Figure 41a Resistance of bacterial strains to copper/silica (Cu/SiO_2) coated surfaces

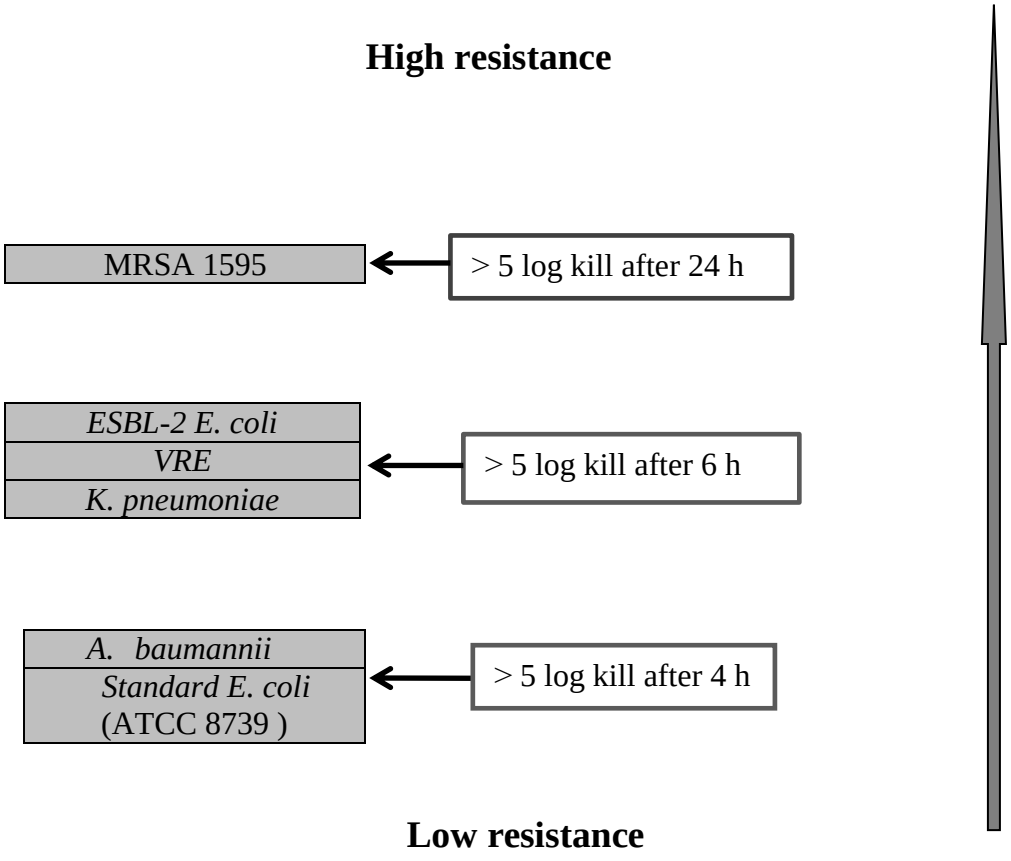

Figure 41b Resistance of bacterial strains to Cu/TiO_2 coated surfaces under UVA.

The MIC of copper

The minimum inhibitory concentration is defined as the lowest concentration of an inhibiting compound at which there was no visible growth. Results from the present study demonstrate that copper had a marked effect on *E. coli* and *S. aureus*. The MIC of copper sulphate was seen in as little as 4 h for both strains (0.5mM for *S. aureus* and 0.25mM for *E. coli*). However, the MIC for *S. aureus* was higher than that of *E. coli*. *S. aureus* is a Gram-positive bacteria and known to resist copper more than the Gram-negative bacteria. Thus, their MIC should logically be higher than that of *E. coli*. Santo and others have reported different MIC of copper for *E. coli* (3.5 mM), which is higher than the MIC finding in this study, but different growth media were used. They suspended the bacteria in NB which is known to affect the copper toxicity (Santo *et al*., 2010). In another study

large variations were observed in the susceptibility of the different bacterial species isolated from livestock to copper. They showed that the Gram-positive *S. aureus* was more susceptible to copper than the Gram-negative *E. coli*. The MIC of twenty-two strains of *S. aureus* was 2 mM, whereas 18 strains had MIC ranging from 8-12 mM. However, *E. coli* species had MIC ranging between 16-20 mM (Aarestrup and Hasman, 2004). In contrast, *E. coli* was more sensitive to killing by copper than *E. faecium* and their MICs were 20mM and 24mM respectively (Elguindi *et al*., 2011). *Bacillus subtilis* species were able to tolerate different concentrations of copper ranging from 1 mM to 3 mM and concentrations of silver ranging from 1 mM to 5 mM (Hookoom and Puchooa, 2013). However, there is insufficient data describing the MIC of copper, particularly on bacterial strains that cause the majority of nosocomial infections in literature reviews. In fact, comparisons between these studies were useless, since different growth media, different copper compounds (e.g., $CuCl_2$), different sizes of bacterial inoculum and different inoculation techniques (dry or wet) were used in different studies. However, all of these factors were not discussed. The killing of *E. coli* on copper surface was enhanced by fluorescent light. A 99.9 % reduction was achieved within 2 h compared to 4 h in the dark. There was a very low level of UVA in the fluorescent light used (<0.01 mW/cm^2) which may have been responsible for the high activity seen in the fluorescent light, However, it is well-known that copper is a semiconductor and when in contact with air is able to form CuO and can be activated by light with a wavelength of <approx. 720 nm (Baghriche *et al*., 2012), leading to surface oxidative radical formation which causes damage to the bacterial cell wall. This can explain the acceleration of the bacterial inactivation of *E. coli* under light with respect to the dark inactivation (Castro *et al*., 2010).

The killing mechanism of copper will be discussed further in the following chapter.

3.3.2 Antimicrobial activity of coated steel surfaces

Stainless steel is commonly used in the food industry and in health-care environments for many reasons, including its ability to be regularly cleaned and its resistance to corrosion. However, there is no essential antimicrobial advantage to using this metal (Grass *et al*., 2011). Many recent studies have tested the antimicrobial activity of copper compared with that of stainless steel. The results showed that there was no antimicrobial effect over 6 h on stainless steel, while on copper complete killing is reached within 60 min (Faúndez *et al*., 2004, Noyce *et al*., 2006a). However, steel with metallic compounds such as silver or copper has shown good antimicrobial activity (Zhang *et al*., 2012b). . In the present study *E. coli, S. aureus*, and *S. enterica Typhimurium* were killed on copper-silica-coated steel surfaces. The killing rate was different on different samples due to different amounts of copper in each sample. The highest killing rate was seen on samples 2C and 5 which had the highest amounts of copper. However, the rate of killing was dependent on both the strain tested and surface type. For example, *E. coli* was completely killed after 24 h on 2C (painted steel) while the survival rate of *S. aureus* and *S. enterica Typhimurium* was decreased by 3 log on the same test sample. However, on 2C (steel) showed 4 log reduction compared to 3 log for *S. aureus* after 24 h of incubation. A similar finding was described in a previous study (Dan *et al*., 2005), where research showed that Cu-implanted on steel had high antimicrobial activity on *E. coli* compared with *S. aureus*. However, the activity against *S. aureus* was increased when tested with surfaces contain higher amounts of Cu ions. The authors suggested that the difference in the antimicrobial effect on different kinds of bacteria is related to two aspects. One is the concentration of Cu ions deposited on the surfaces and the other is the influence of the antibacterial mechanism. Furthermore, Cu ions released from the surfaces are absorbed onto the surface of bacteria cells, which leads to the damage of cell membranes and thus other cell molecules such as proteins. Therefore, the ability of Cu ions to penetrate the cell membrane is a fatal factor. (Dan *et al*., 2005). However, this was in contrast with a

recent study. The Cu modified steel showed good antimicrobial ability against the Gram-negative *E. coli* and the Gram-positive *S. aureus*. The viability of *E. coli* and *S. aureus* was reduced within 1 h by over 99% and 97% respectively, and 100% killing occurred for both strains after 3 h of exposure to Cu modified steel (Zhang *et al*., 2012b). The contradiction results reported by the above studies could be due to the surfaces structure as result of different production methods.

Surface topography

It has been reported that surface roughness has a strong effect in terms of fouling and clean-ability. A value of 0.8 µm has been ascribed to a hygienic stainless steel surface, and surfaces with values above that level would be hard to clean (Whitehead *et al*., 2004). In this study the surfaces roughness was not studied and the retention of bacteria on the surfaces was analysed by stained and observation of the surface under microscope. Despite the fact that there are a number of studies that have been conducted on the effect of surface roughness on microbial retention, some of them contradict one another. Morgan and Wilson (2001) reported that the surface roughness of denture acrylic (artificial teeth replacement) had a direct effect on microbial adhesion on the surface. They showed that the number of bacteria (*Streptococcus oralis*) colonising the surfaces increased with increased surface roughness. Contradicting results were reported in recent study conducted by Page *et al* (2011). They found that there was no direct correlation between surface roughness and the level of microbial adhesion, since the degree of the surface roughness used was smaller than the typical dimensions of a rod shaped prokaryotic cell (1-5µm long by 1µm wide) (Page *et al*., 2011). The different results produced by Page *et al.,* 2011 may was due to the roughness of the surfaces being used which may have been the same or larger than the size of the microbes tested.

The killing rate of steel coated surfaces was much slower than the killing on copper-silica coated glass surfaces. However, comparison between the activities of the two coatings was not applicable, since different preparation methods and different substrates (glass and steel) were used and painted steel has triple layers (silica, copper , silica) compared to co-deposited silica and copper in CVD Salford University samples.

Chapter 4

Physical properties and durability of coated surfaces

4.1 Introduction

Copper (Cu) as an effective antimicrobial material has received extensive attention during the past few years. Numerous scientific studies have reported the effective use of copper and its alloys in reducing the number of pathogenic bacteria. Copper has multi-toxicity against different microorganisms including multidrug resistant bacteria such as MRSA (Methicillin resistant *Staphylococcus aureus*) and carbapenem-resistant bacteria. Despite the fact that details of the antimicrobial mechanisms of these materials are still under investigation, it is clear that several mechanisms are involved. Recent findings suggest that the direct contact between bacteria and metallic copper is an important factor in contact killing, which could induce severe damage of the bacterial envelope and might constitute a primary killing mechanism. In addition, copper ions dissolved from the copper surfaces play a major role (Hans *et al*., 2013). There are many reports in the literature supporting the role of copper ions in copper toxicity but some of them contradict each other. Most of them agree on the point that membrane and DNA damage occurs as a result of the copper's effects; however, which mechanism occurs first is still under debate (Santo *et al*., 2012). Thus it is important to learn if certain membrane-bound proteins or membrane lipids themselves are the main targets for the copper in cells and whether the damage to these specific targets leads to the rapid bacteria killing on a metallic copper surface (Wei *et al*., 2014).

A successful biocide film must provide the most toxic environment possible to bacteria but, at the same time, remain harmless to humans. It must maintain its biocide activity over their expected lifetimes, which include regular cleaning methods. Durability and adhesion of coatings on surfaces are the most important factors for producing long-life biocide surfaces. Indeed, the incorporation of

silica in metal coating means that the lifetime and stability of the coating could be strongly enhanced (Cook *et al*., 2011). Colourless and durable materials that slowly release silver ions over a long period are required for medical applications (Kawashita *et al*., 2000).

The main problem with Cu surfaces is that they are relatively soft and easily abraded metals which can lead to increased microbial contamination (Airey and Verran, 2007). In this work these limitations were addressed by combining the copper with silica, co-deposited by flame-assisted chemical vapour deposition (FACVD) which produces a hard, glass-like surface which retains antimicrobial activity. The copper is mainly in the form of CuO. In this chapter the properties and durability of the coatings is reported.

4.2 Results

4.2.1 Appearance and durability of the coated glass and tiles

4.2.1.1 Appearance of the coated surfaces

4.2.1.1.1 Visual appearance

The visual appearance of Cu/SiO_2, Ag/SiO_2 and Cu/TiO_2 films on glass is shown in Figure 42(a) and coated tiles of Cu/SiO_2 and Ag/SiO_2 is shown in Figure 42 (b). Both films Cu/SiO_2 and Ag/SiO_2 had a pale brown tinge on both substrates (glass & tiles) which was darker in the films with a higher Ag content (Figures 42 b & c). The darkness of films with higher content of Ag is clearer on the full sized coated tiles (Figure 42c), whereas the Cu/TiO_2 film had a darker transparent pale brown colour. Transmissions of glass coated Cu/SiO_2, Ag/SiO_2 and Cu/TiO_2 in the visible range of 400nm-700nm were 90.6, 84.5-88.5 and 73.7 respectively compared to 91.5% for the control glass.

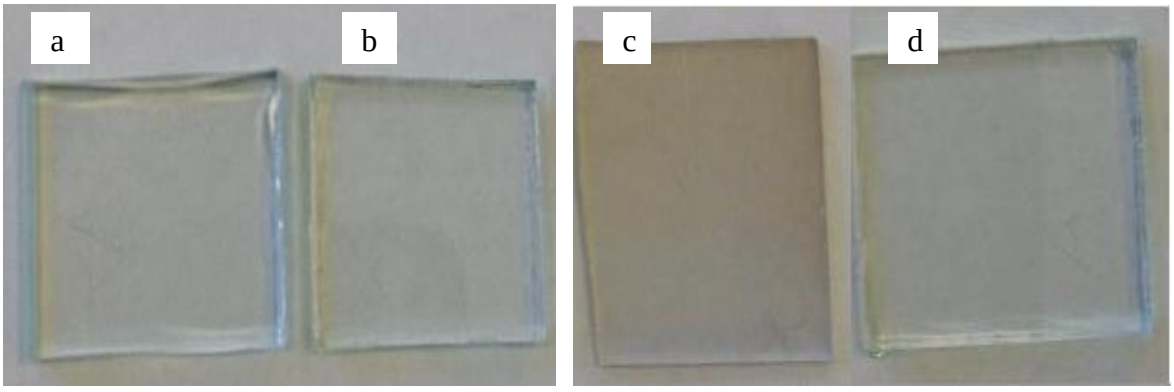

Figure 42a Appearance of coatings on glass, (a) Cu/SiO_2, (b) Ag/SiO_2, (c) Cu/TiO_2, and (d) control glass

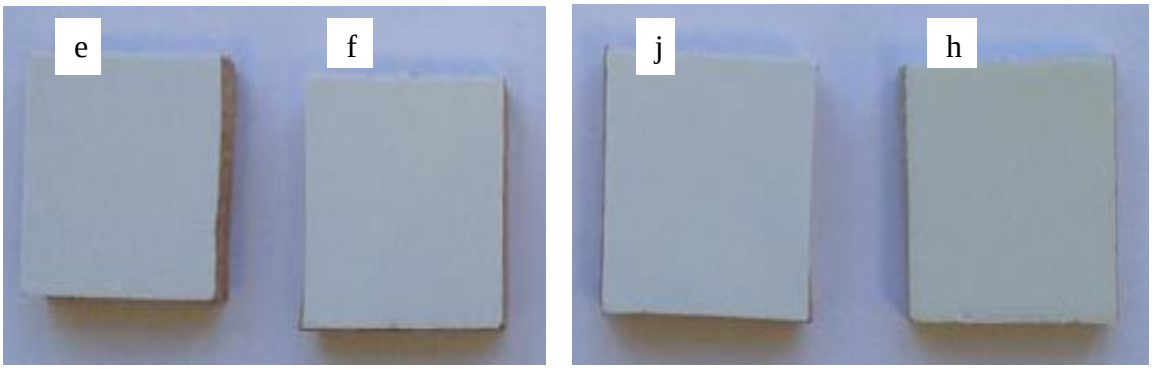

Figure 42b Appearance of coatings on ceramic tiles, (e) Cu/SiO_2, (f) control tile, (j) Ag/SiO_2 (0.05 M), and (h) Ag/SiO_2 (0.25 M)

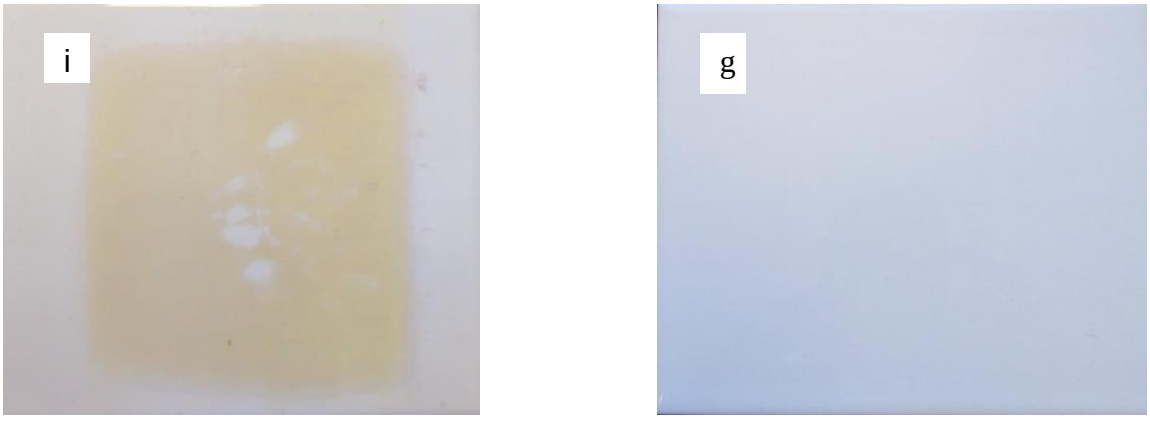

Figure 42c Appearance of full size coatings on ceramic tiles, (i) Ag/SiO_2 (0.25 M) and (g) Cu/SiO_2 (0.25 M)

4.2.1.1.2 Surface morphology

Surface morphology of all coated surfaces was studied by SEM (Figure 43a and 43b) all coated samples (glass and tiles) showed nano-structured features within an amorphous background with evenly distributed aggregates embedded in the surfaces. However the glass showed higher numbers of aggregates than the tiles and this may due to the glossy glazed surfaces of the tiles. The results suggest that the copper and silver aggregates grow by forming islands in the silica. With higher concentrations of silver, islands coalesce and may reduce the ability of silica to bind the coating onto the substrate.

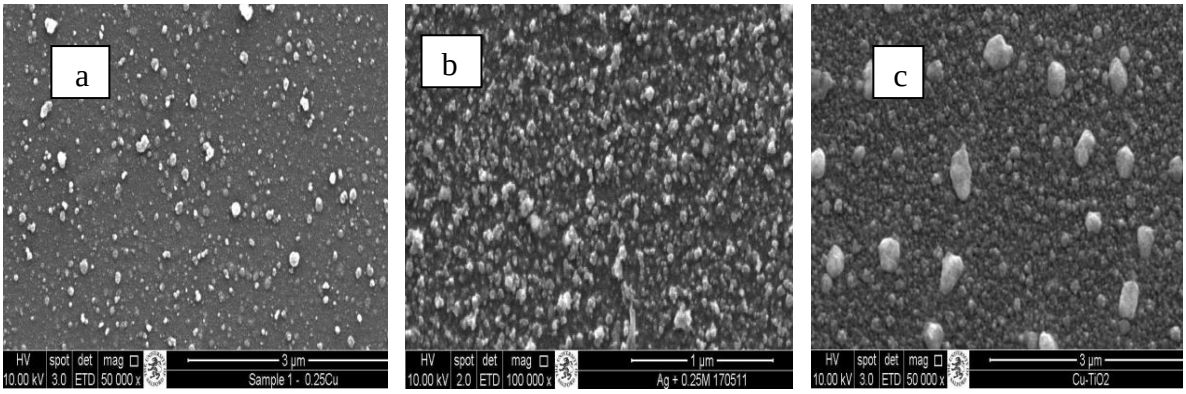

Figure 43a SEM of coated glass samples, (a) Cu/SiO_2, (b) Ag/SiO_2, and (c) Cu/TiO_2

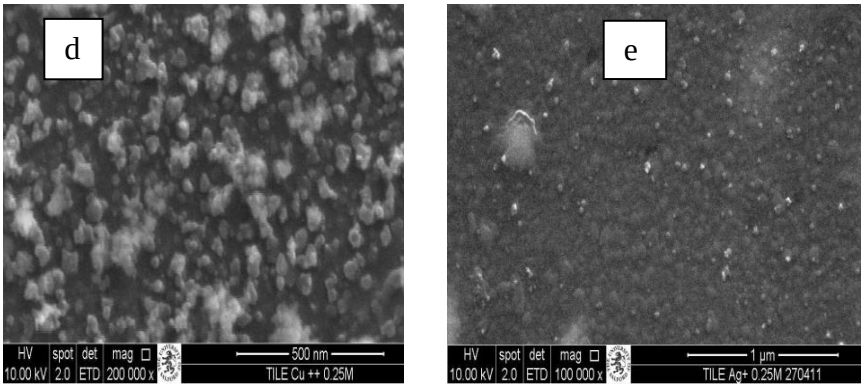

Figure 43b SEM of coated tiles samples, (d) Cu/SiO_2 and (e) Ag/SiO_2

(In order to get clear figures different magnification were used depending on surface coatings)

4.3.2 Durability

4.3.2.1 Hardness of films (glass and tiles substrate)

The Mohs hardness of glass films were 5.6 for Cu/SiO_2 coating, 5.9 for Ag/SiO_2 (0.05m) and 5.6 for Cu/TiO_2 which giving a hardness comparison to stainless steel for (stainless steel = 5.5). However the Mohs hardness decreased with increasing Ag content (0.25 M) and was 2.3. The Mohs hardness

of coated tiles were less than the hardness of glass coated surfaces and were 3.2 and 2.7 for copper/ silica and silver/ silica respectively but this again may be related to the glazing.

4.3.2.2 Tape test

All coated films Cu/SiO_2 (glass and tiles) and Cu/TiO_2 remained intact in the Scotch tape test showing a good adhesion to the substrates. However, the hardness of Ag/SiO_2 coating, at high concentration of Ag (0.25 M) decreased and some of the coating was lost on the tape test as shown above in Figure 42(c), whereas coatings with the lower concentration of Ag passed the tape test.

4.3.2.3 The effect of low, medium and high washing on antimicrobial activity of Cu/SiO_2 coated tiles.

The substrate size used for the British Standard test (BS) for antimicrobial hard surfaces was 2 cm square; however the appropriate substrate size for the washability test machine was bigger than this size (9 x 5 cm). Therefore it was not possible to follow the BS test and a modified method was created (spray method) and followed as described in chapter 2 (8.1.2).

Preliminary tests showed that the amount of copper in the coatings on tiles affected the durability. Tiles with approx. 0.01at.% Cu lost 50% of the copper with only 1000 rubs of a soft-sponge whereas those with only 0.002 at.% Cu only lost 30% of the copper and it took a further 3500 rubs with an abrasive sponge to remove 75%. However, tiles with the higher amount of Cu were used for this test as they had shown excellent antimicrobial activity. Three washing regimes were chosen to remove low, medium and high amounts of coating. At zero time, the concentration of bacteria found on tested tiles was similar on all tiles (control, before and after wash). All the coated tiles still showed antimicrobial activity after having been washed as shown in figures 44, 45 and 46. The differences

between the control and test (washed tile) of low, medium and high washed tiles were significant up to 24 h ($P \leq 0.05$).

On unwashed coated tiles which had a copper content of approx. 0.009 mass % (Figure 47) complete killing was achieved after 4 h. However, the activity was decreased after washing. With low wash conditions (100 cycles of an abrasive sponge without additional weight) this reduced the copper content by 36% but still gave a > 5 log reduction after 6 h (Figure 44). With medium wash conditions (a further 500 cycles with abrasive sponge and a 335 g weight) the copper content was reduced by a further 26% and there was only a 2.5 log reduction after 6 h increasing to 3 log after 24 h (Figure 45). With high wash (a further 400 cycles of abrasive sponge with a 335 g weight) the copper content was reduced by a total of 75% (Figure 47) and there was only 2 log reduction after 24 h (Figure 46).

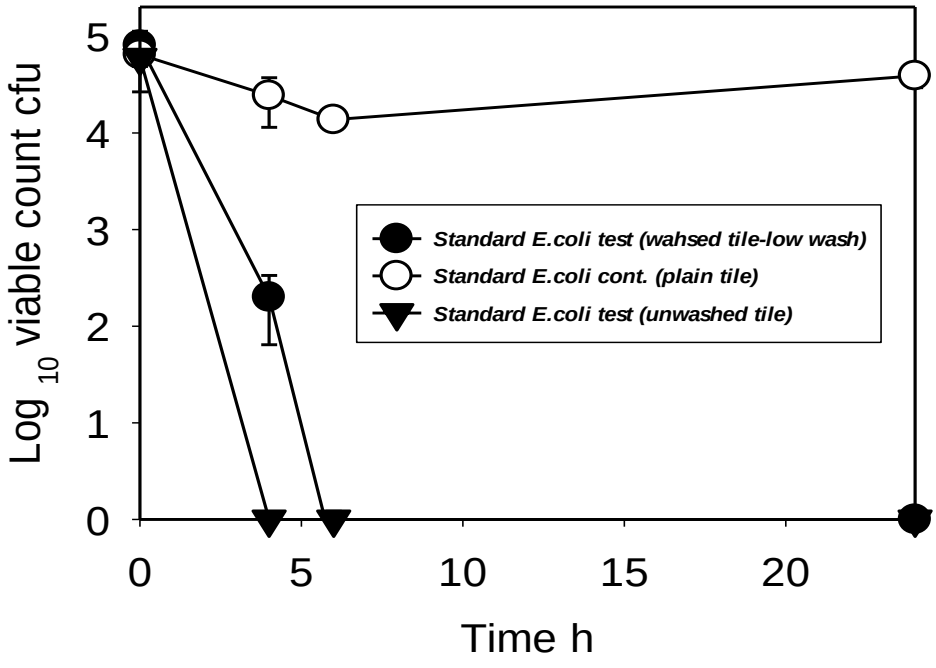

Figure 44 Killing of *E. coli* (ATCC8739) on low washed coated tile Cu/SiO_2 (test), unwashed coated Cu/SiO_2 tile (control test) and uncoated tile (plain control).

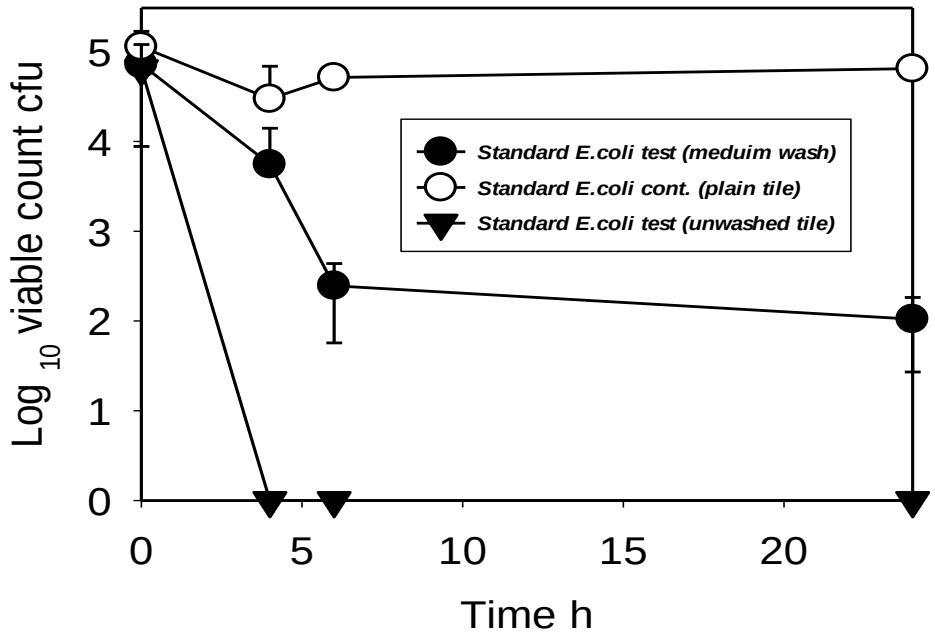

Figure 45 Killing of *E. coli* (ATCC8739) on medium washed coated tile Cu/SiO_2 (test), un-washed coated Cu/SiO_2 tile (control test) and uncoated tile (plain control).

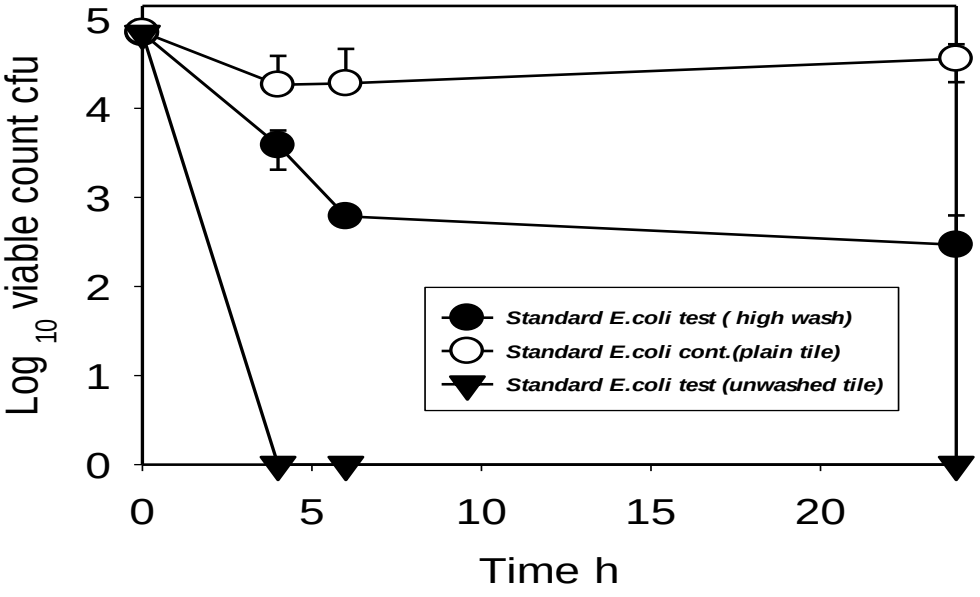

Figure 46 Killing of *E. coli* (ATCC8739) on high washed coated tile Cu/SiO_2 (test), un-washed coated Cu/SiO_2 tile (control test) and un coated tile (plain control).

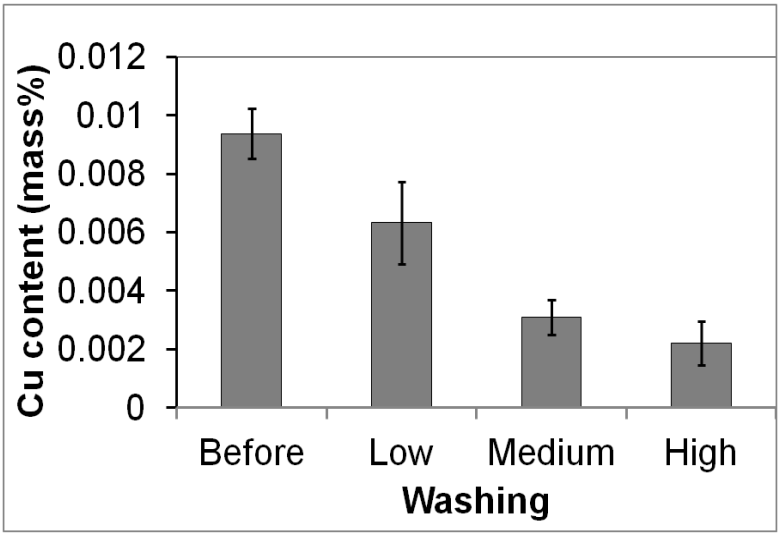

Figure 47 The Cu content (mass %) of different level of washed tiles (low, medium and high wash) and un-washed tiles

4.3.3 The effect of temperature on antimicrobial activity of copper

4.3.3.1 Antimicrobial activity of Cu/SiO_2 at 35°C

The BS ISO 22196:2009 & 2011 specifies a temperature of 35°C. The coated film was more active against both types of bacteria Gram-positive and Gram-negative at 35°C compared to the activity at 25°C (room temperature, chapter 3-figure 16). A >5 log kill (>99.999%) was achieved within 1 h for standard *E. coli* and after 2 h for standard *S. aureus* on test surfaces and 3 log reduction on control glass (Figure 48). MRSA 15 was more resistant to the copper at 25°C (chapter 3-Figure 29) however at 35°C a > 4 log reduction was reached within 2 h increasing to >5 log reduction after 4 h as shown in Figure 49. However there was also a reduction in the controls for EMRSA15 (Figure 49). There were no viable cells in either test or controls after 24 h with the standard strain of *S. aureus* (data not shown).

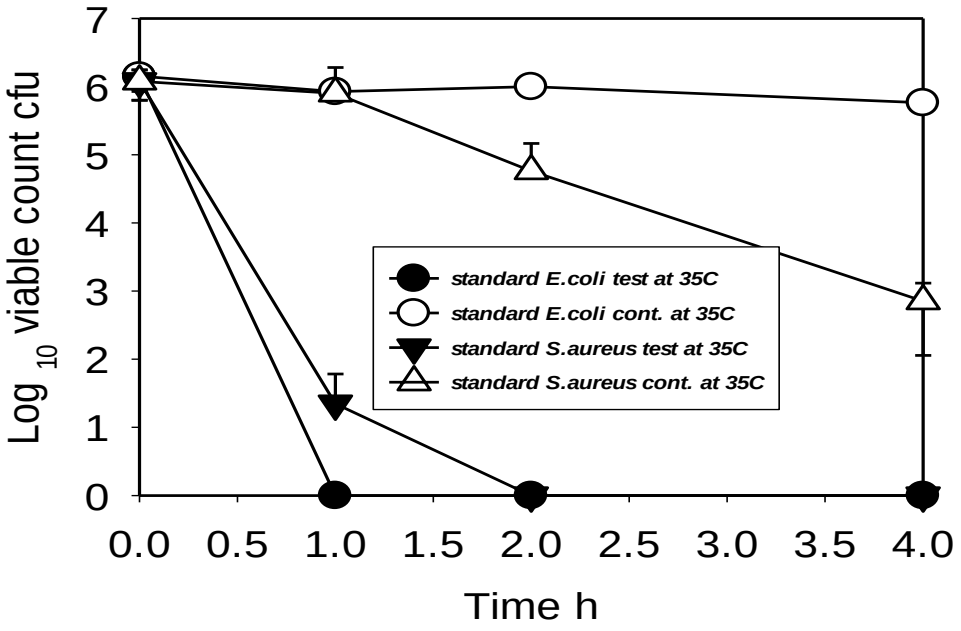

Figure 48 Killing of standard *E. coli* (ATCC8739) and standard *S. aureus* (ATCC 6538) on Cu/SiO_2 coated glass (test) and uncoated glass (control) at 35°C

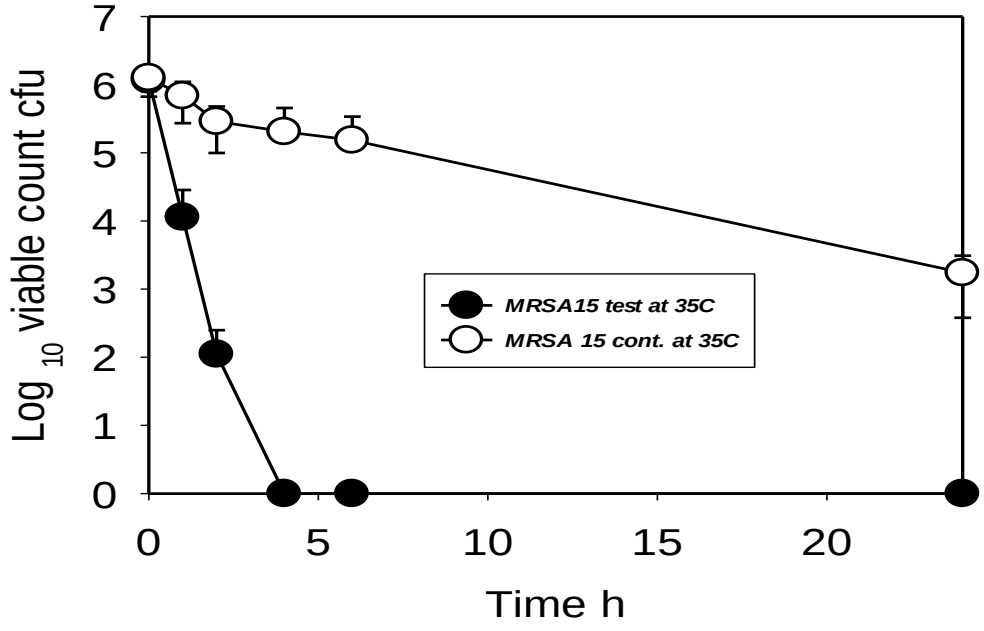

Figure 49 Killing of MRSA15 on Cu/SiO_2 coated glass (test) and uncoated glass (control) at 35°C.

4.3.3.2 Antimicrobial activity of coated surfaces at 5°C

4.3.3.2.1 Antimicrobial activity of Cu/SiO_2

In order to test for potential applications at low temperatures e.g. food storage the activity of coated film at 5°C was determined. The rate of killing was lower at 5°C compared to activity at higher temperatures (at 35°C Fig.48 and at 25°C Fig. 25-chapter 3) with only a 2.5 log reduction after 48 h increasing to >5 log reduction after 72 h for standard *E. coli* and only 1.9 log reduction after 48 h increasing to 3 log after 72 h for standard *S. aureus* (Figure 50). There was a similar activity against *ESBL*$^+$ *E .coli* and *A. baumannii* with only 2 log reduction after 24 h (Figure 51).

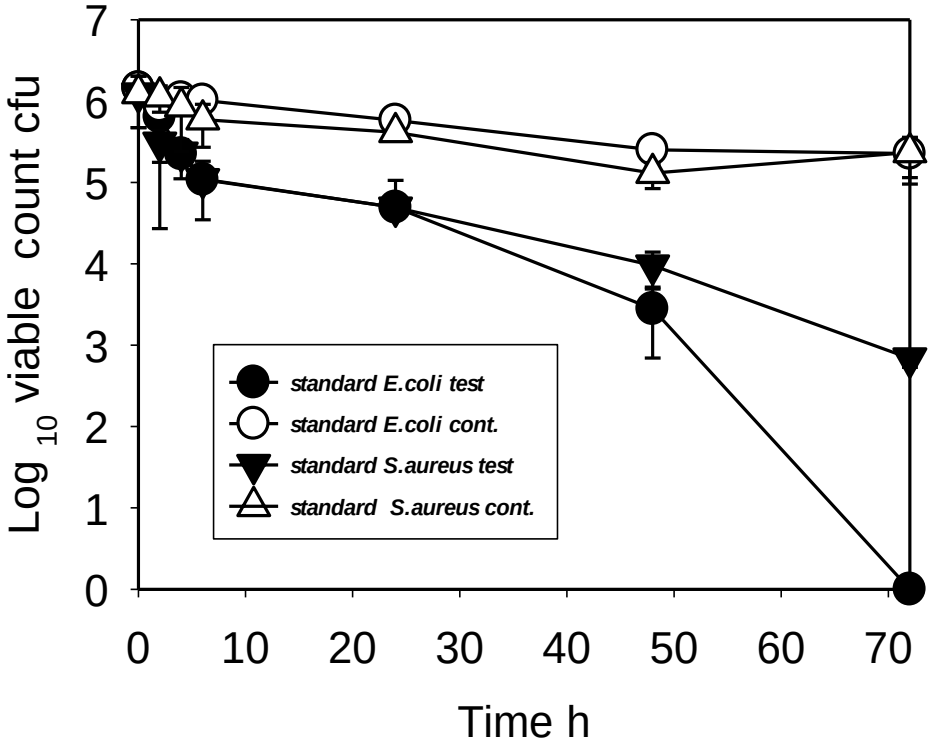

Figure 50 Killing of standard *E. coli* (ATCC8739) and standard *S. aureus* (ATCC 6538) on Cu/SiO_2 coated glass (test) and uncoated glass (control) at 5ºC.

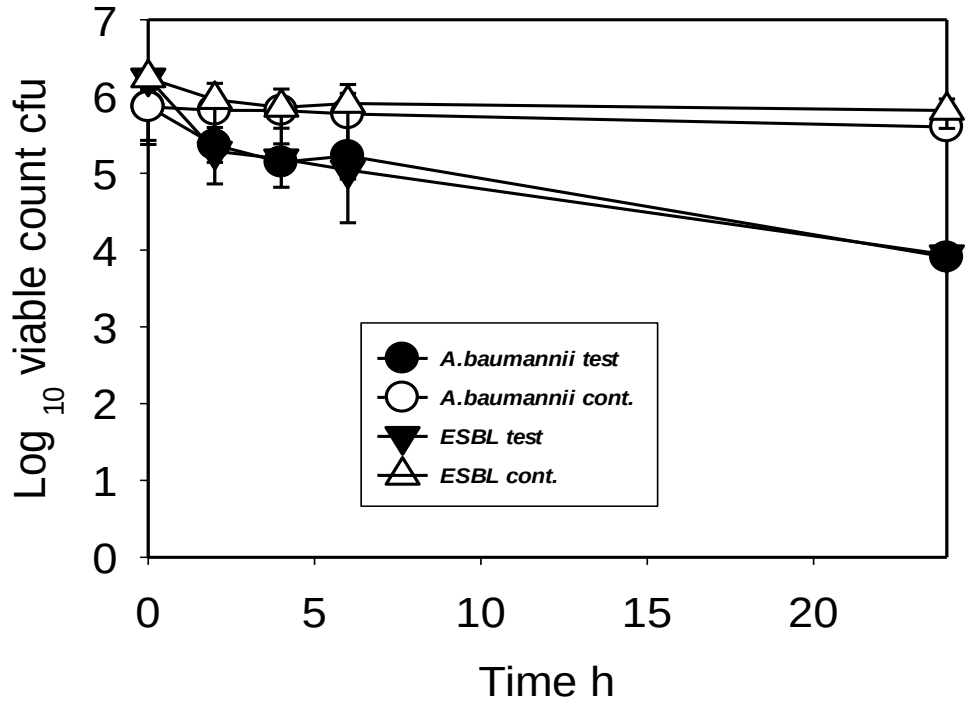

Figure 51 Killing of *A. baumannii* and *ESBL E. coli* on Cu/SiO_2 coated glass (test) and uncoated glass (control) at 5ºC.

4.3.3.2.2 Antimicrobial activity of Cu/TiO_2

The killing curve of $ESBL^+$ strain of *E. coli* at 5°C is shown in Figure 52. The activity against the $ESBL^+$ *E. coli* was reduced after 4 h compared to incubation at room temperature (chapter 3; fig. 11) with only a 1.1 log kill but still gave a > 5 log kill after 6 h under UVA light. *A. baumannii* was more sensitive with a 3.3 log kill after 4 h increasing to >5 log kill after 6 h (Figure 43). The reduction in activity was much greater with UVA illumination with a >5 log reduction after 6 h but only 2.8 log reduction with fluorescent light on ESBL *E. coli* and 1.8 log on *A. baumannii*.

Generally the inhibitory effect of the coatings was clearly less active at low temperatures compared to higher temperature as shown in chapter 3 (Figures 11 and 13).

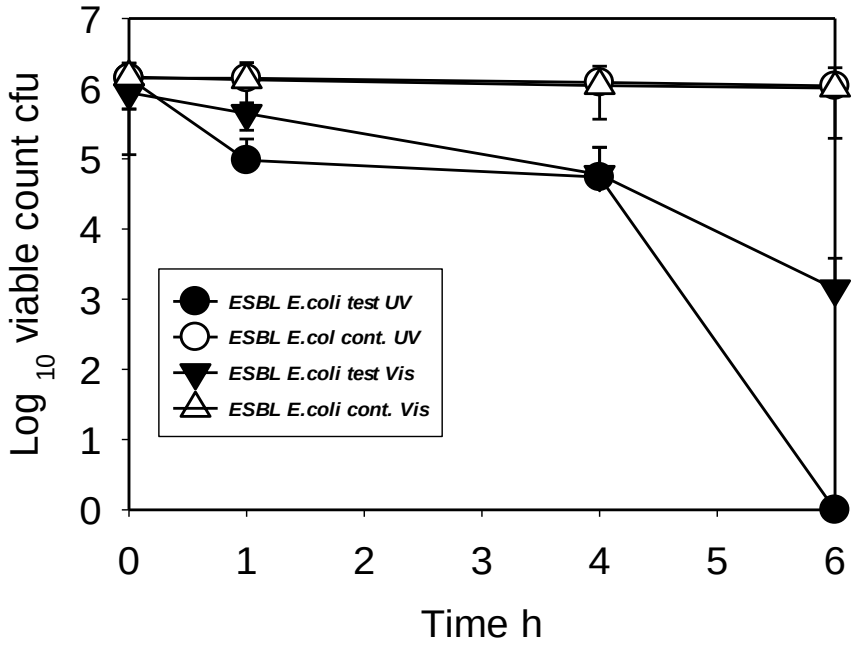

Figure 52 Killing of *ESBL*$^{+}$ *E. coli* with different illumination (UVA and fluorescent light) at 5°C on Cu /TiO_2 coated glass (test) and uncoated glass (control).

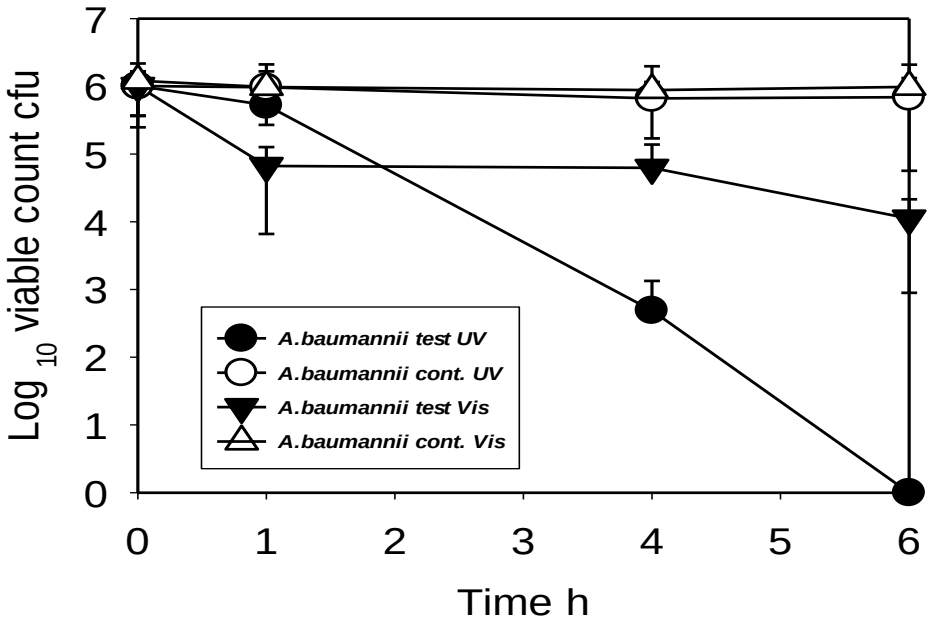

Figure 53 Killing of *A. baumannii* with different illumination (UVA and fluorescent light) at 5°C on Cu /TiO_2 coated glass (test) and uncoated glass (control).

4.3.4 Killing mechanism of copper

4.3.4.1 The effect of copper on bacteria cell membrane

The cells viability of standard *E. coli* and standard *S. aureus* stained with propidium iodide and Syto 9 are shown in figure 55-56 (a-c) and 57-58 (a-c) respectively. Cells with damaged membranes appear red (propidium iodide), and viable cells (un-damaged membranes) stained green (Syto 9). Membrane damage suggested the cells were dead. The results show that all *E. coli* and *S. aureus* cells on the control surfaces gave only green fluorescence, indicating that all bacteria had intact cell membranes as shown in figures 55a-c and 57a-c. However, no viable *E. coli* cells were detectable on test surfaces after 4 h (Figure 56 a-c) which showed that these surfaces killed 99.999% (>5 log

reduction) of *E. coli* within 4 h. The increase in number of red cells (damaged) correlated well with killing kinetics curve for both strains as shown below (Figure 54). Similar results were detected for *S. aureus* but viable cells (green cells, Figure 57 a-c) were still detectable up to 6 h despite the viable count being reduced by 2.3 log (>99% kill). More than 99.99% of dead cells were seen after 24 h (Figure 54). Thus cells were mostly red showing that the membrane has been damage.

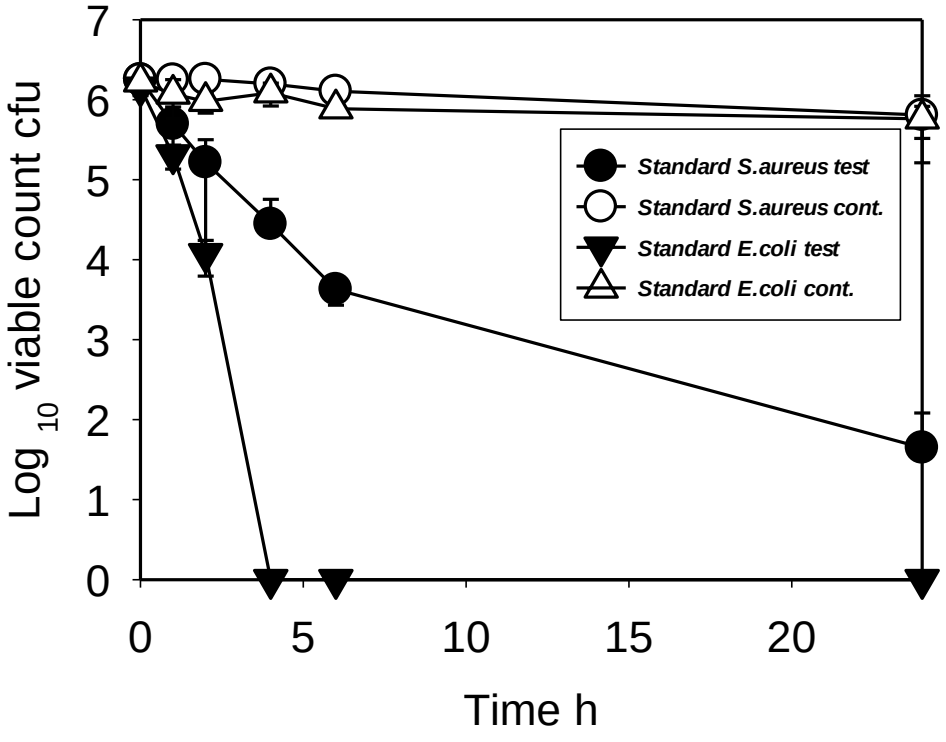

Figure 54 Killing of standard *E. coli* and Standard *S. aureus* on Cu/SiO_2 coated glass (test) and uncoated glass (control)

0h-C **1h-C**

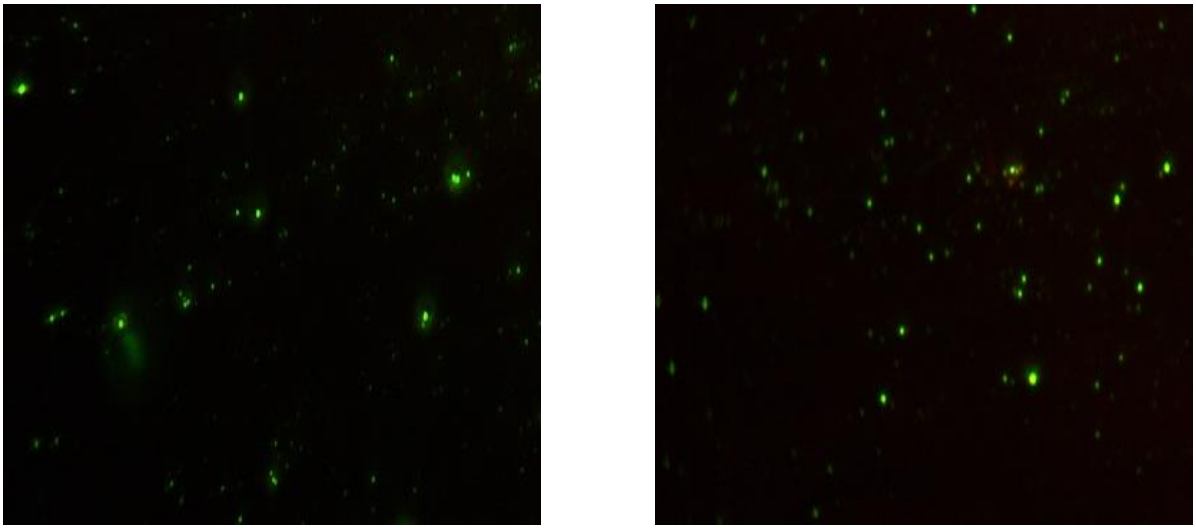

Figure 55a Shows live and dead cells of standard *E. coli* on control surfaces after 0 and 1h.

2h-C **4 h-C**

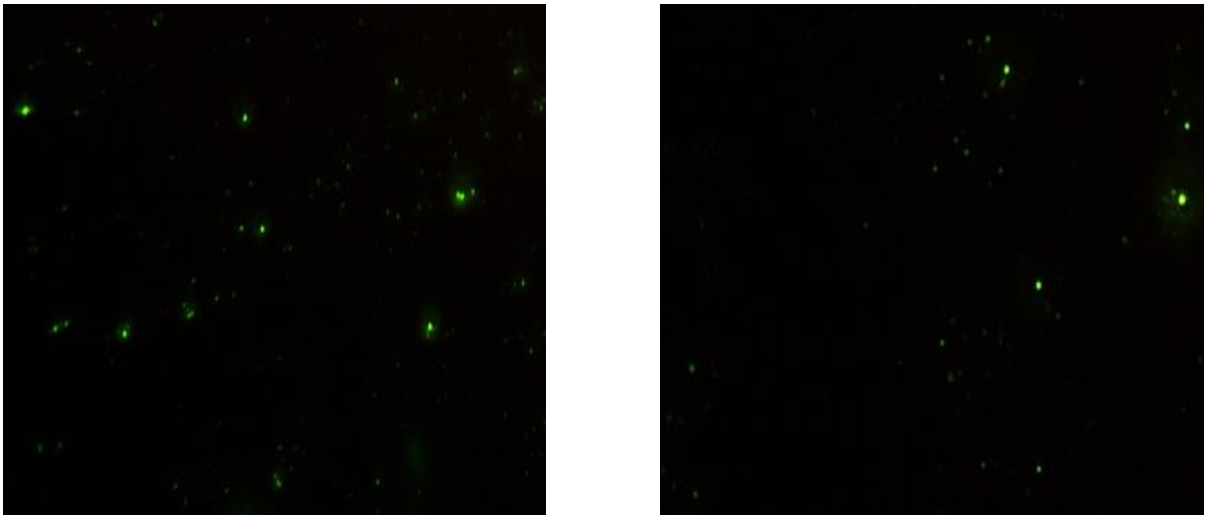

Figure 55b Shows live and dead cells of standard *E. coli* on control surfaces after 2 and 4 h.

6h-C **24h-C**

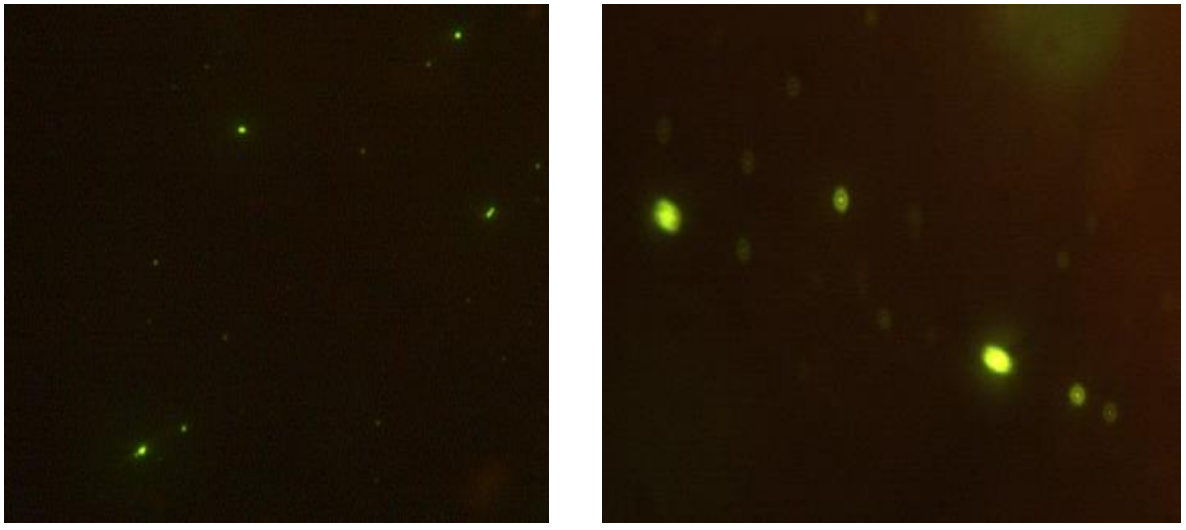

Figure 55c Shows live and dead cells of standard *E. coli* on control surfaces after 6 and 24h. (The big green pigment is stain reflection on slides not bacterial cells)

0h-T **1h-T**

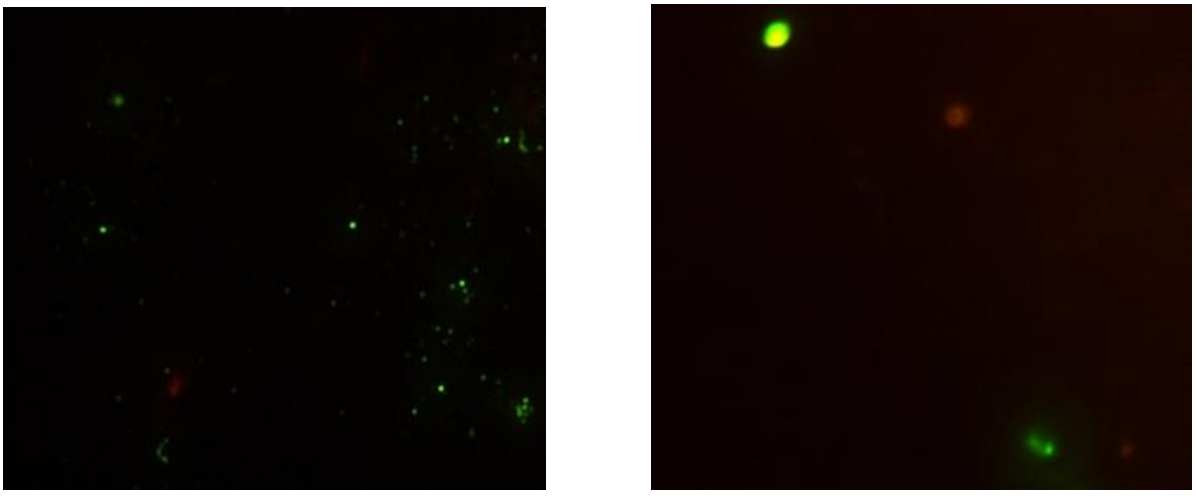

Figure 56a Shows live and dead cells of standard *E. coli* on coated surfaces (Cu/SiO_2 –test) after 0 and 1h

2h-T **4h-T**

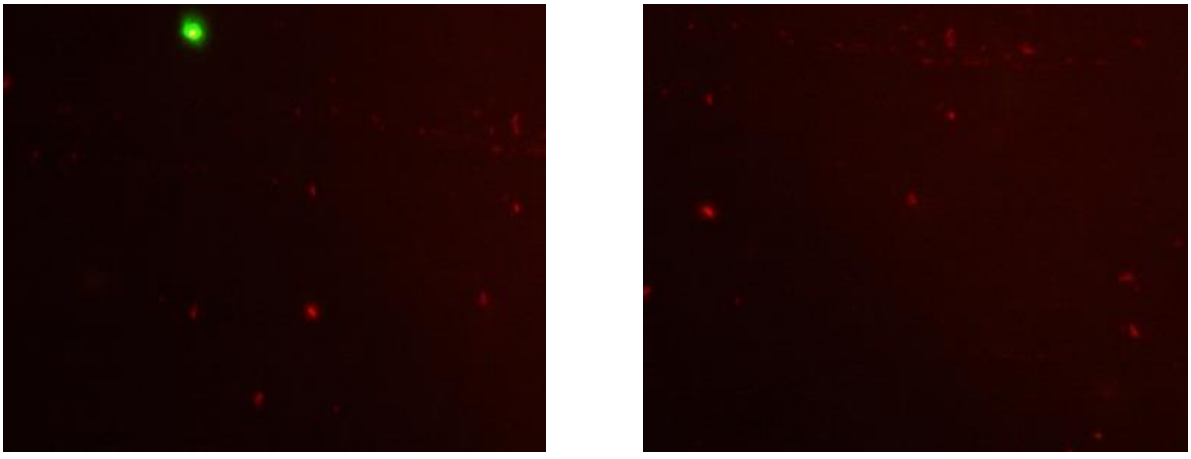

Figure 56b Shows live and dead cells of standard *E. coli* on coated surfaces (Cu/SiO_2 –test) after 2 and 4h

6h-T **24h-T**

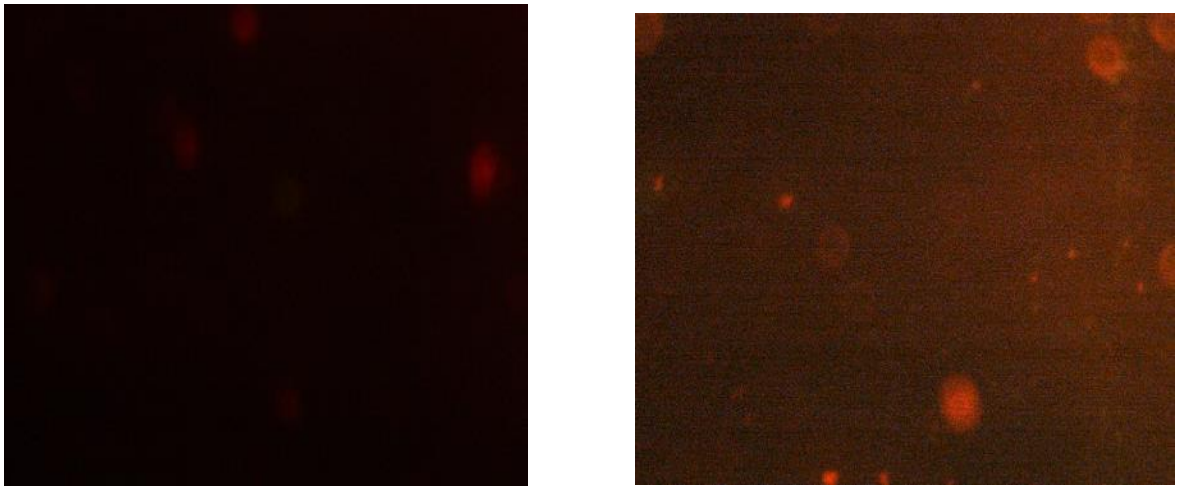

Figure 56c Shows live and dead cells of standard *E. coli* on coated surfaces (Cu/SiO_2 –test) after 6 and 24h.

0h-C **1h- C**

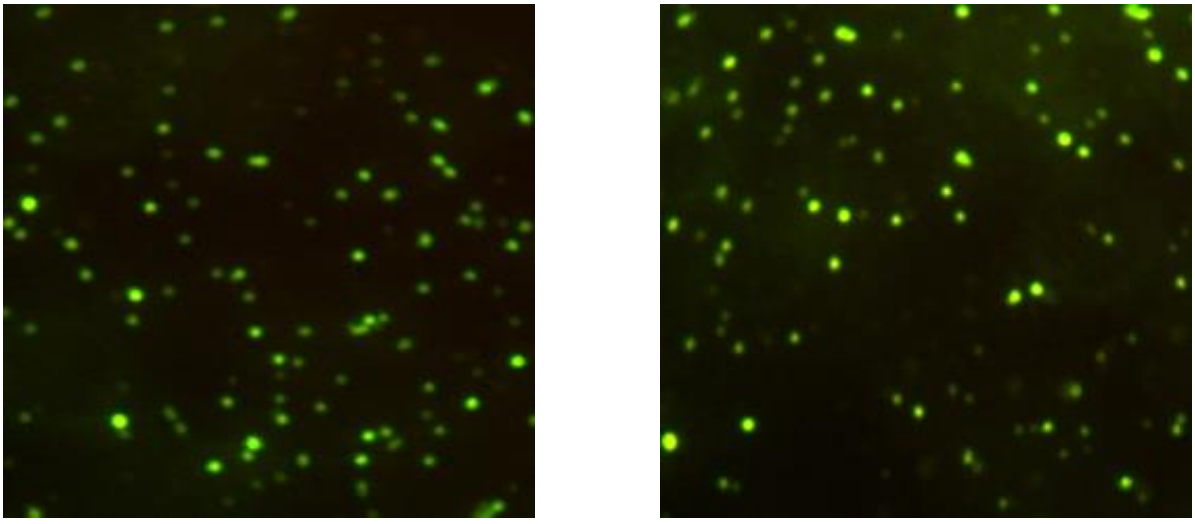

Figure 57a Shows live and dead cells of standard *S. aureus* on control surfaces after 0 and 1h.

2h-C **4h-C**

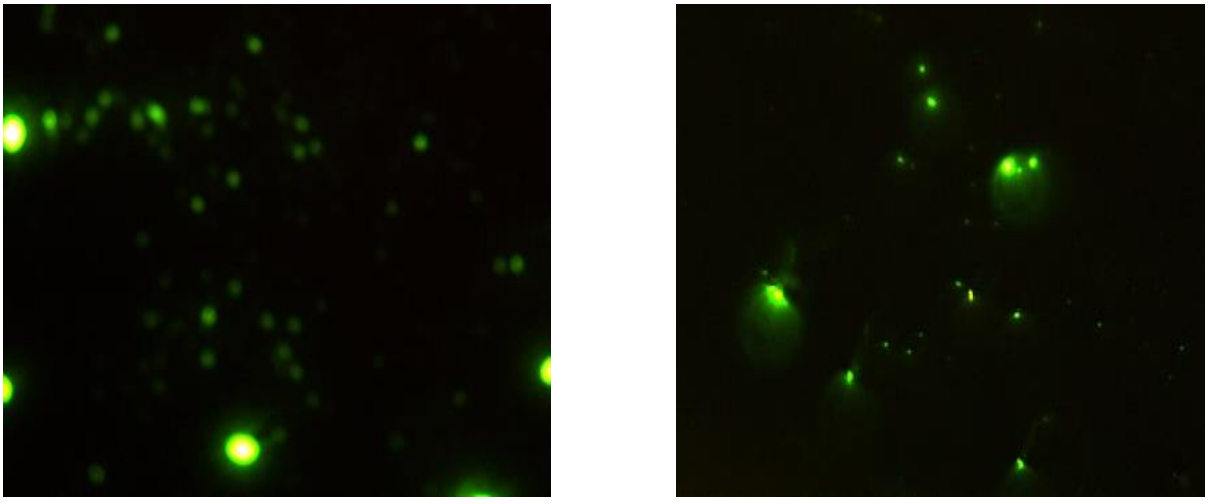

Figure 57b Shows live and dead cells of standard *S. aureus* on control surfaces after 2 and 4h.

6h-C **24h-C**

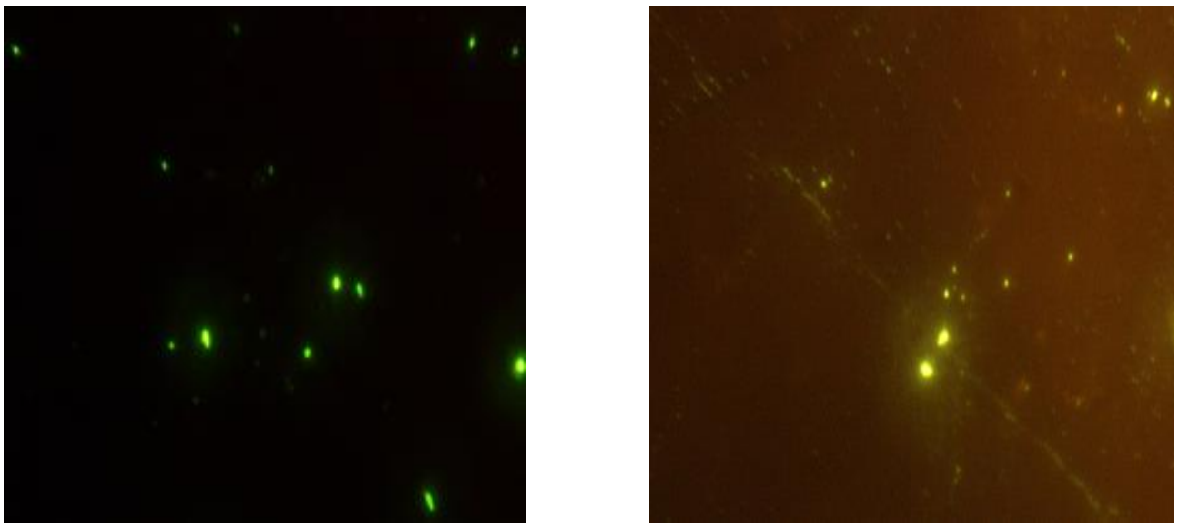

Figure 57c Shows live and dead cells of standard *S. aureus* on control surfaces after 6 and 24h.

0h-T **1h-T**

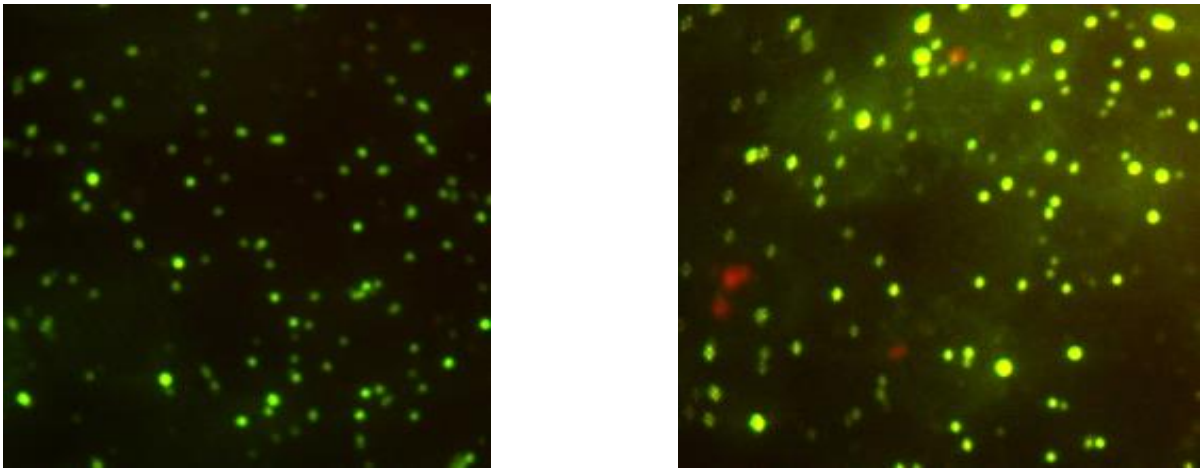

Figure 58a live and dead cells of standard *S. aureus* on coating surfaces (Cu/SiO_2 - test) after 0 and 1 h.

2h-T **4h-T**

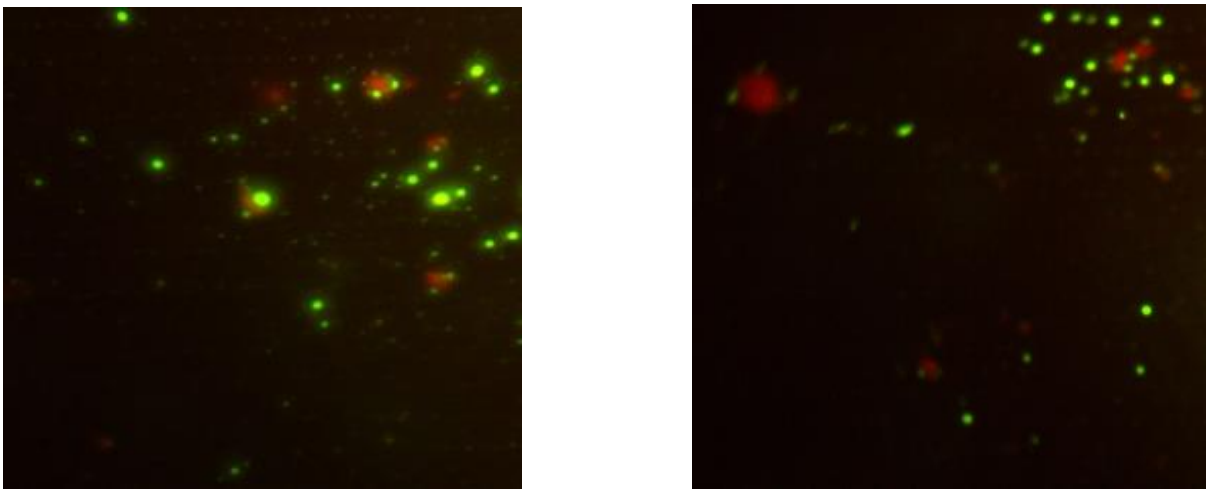

Figure 58b Shows live and dead cells of standard *S. aureus* on coated surfaces (Cu/SiO_2 -test) after 2 and 4 h.

6h-T **24h-T**

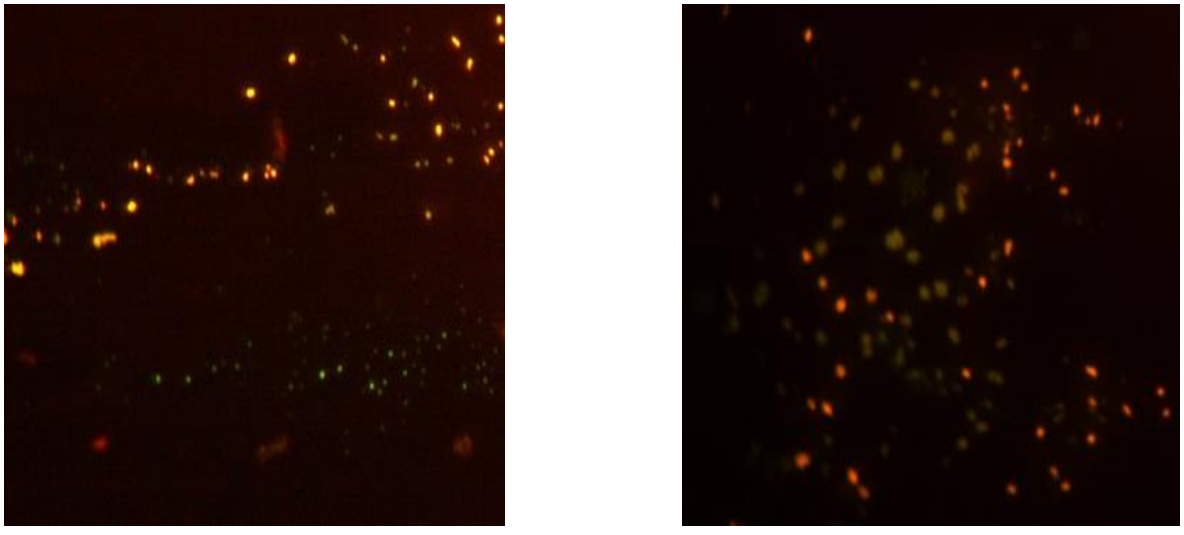

Figure 58c Shows live and dead cells of standard *S. aureus* on coated surfaces (Cu/SiO_2 –test) after 6 and 24h.

4.3.4.2 The effect of copper on bacterial DNA

The comet assay was performed after 0 and 4 h of exposure to copper and the comet tails or DNA breaks were visualized by fluorescence microscopy. The *E. coli* and *S. aureus* cells that were exposed to copper surfaces are shown in Figures 59 and 60. The *E. coli* showed DNA damage after 4 h compared to the control. In contrast, no comet tails or DNA fragmentation was observed in *S. aureus* after 4 h of exposure on both test and control surfaces (Figure 60) despite a 1.5 log (>95%) kill after this time (Figure 54).

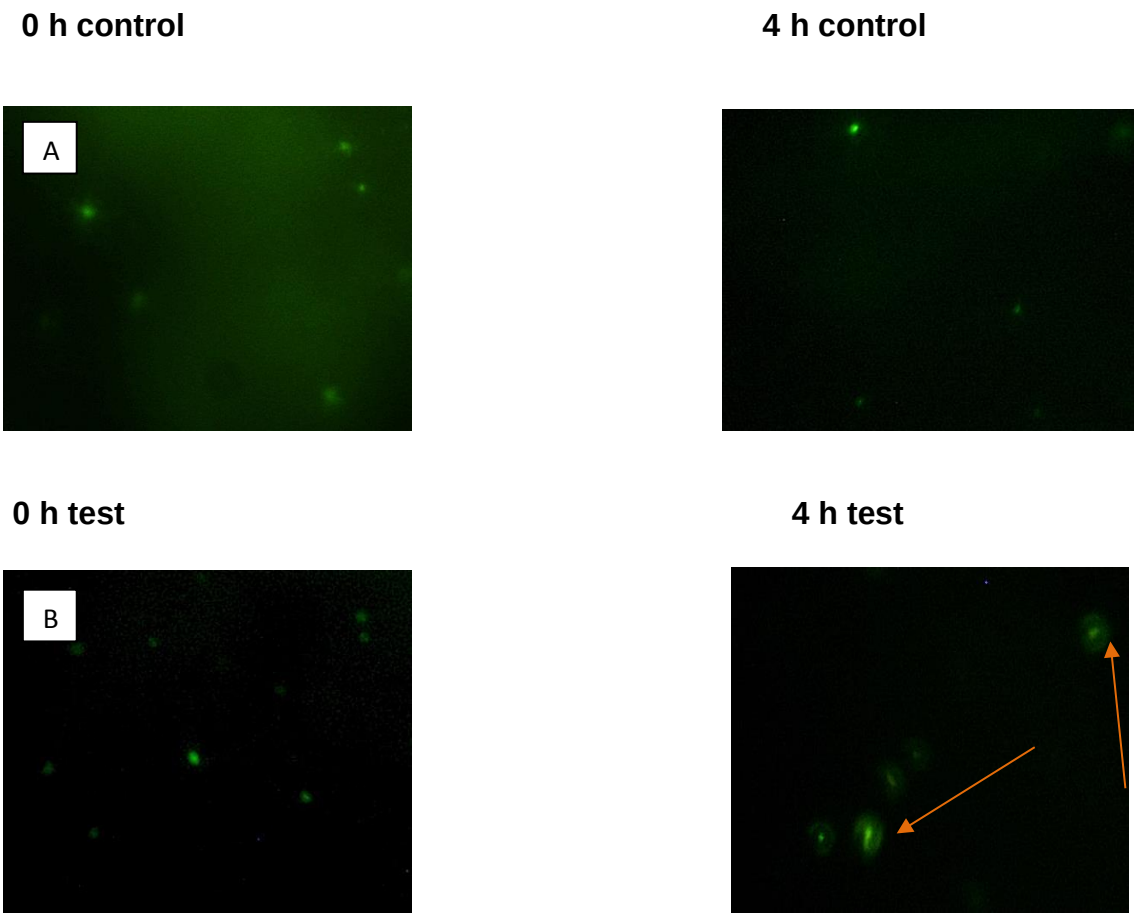

Figure 59 DNA damage of standard *E. coli* on (A) uncoated glass (control) and on (B) test surfaces (Cu/SiO_2 coated glass) after 0 h and 4h. Arrows show the DNA comet migrates from the DNA.

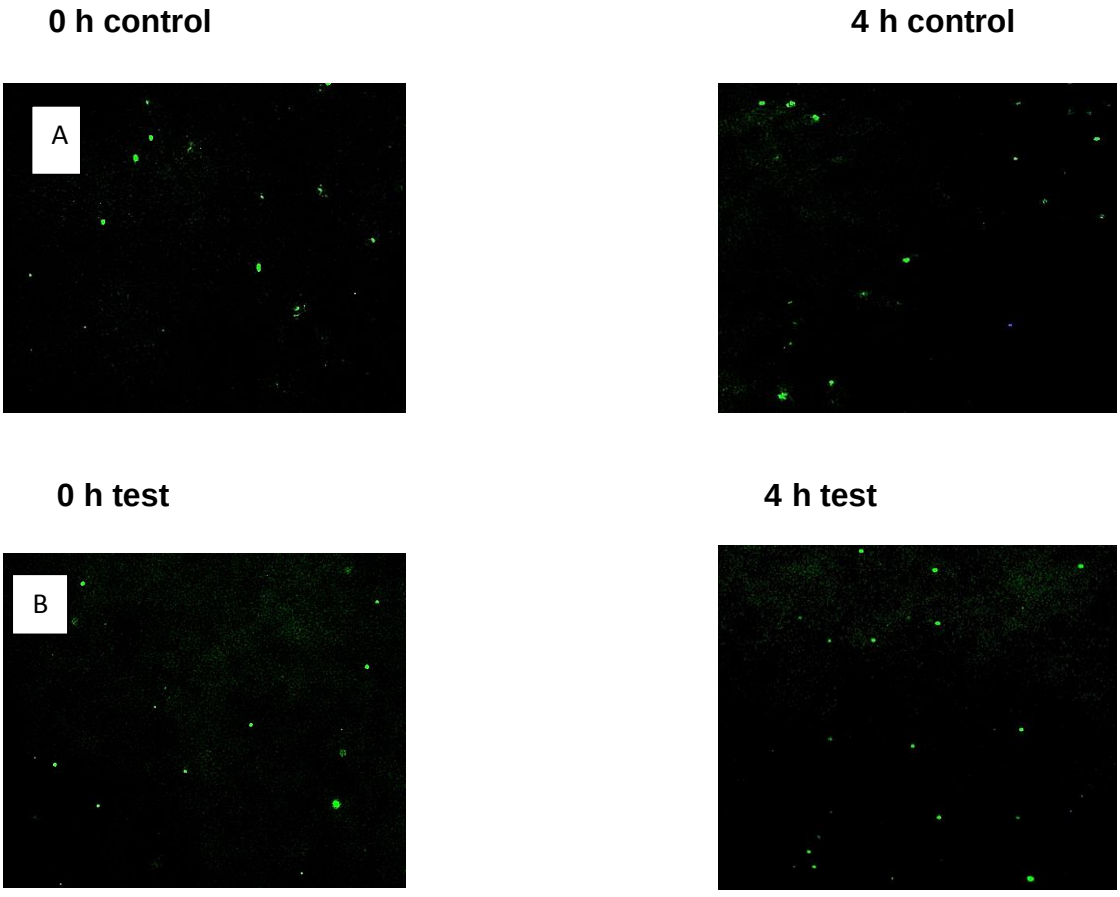

Figure 60 DNA damage of standard *S. aureus* on (A) uncoated glass (control) and on (B) test surfaces (Cu/SiO_2 coated glass) after 0 h and 4h

4.3 Discussion

The coated materials generally had a pale brown or greenish-brown colour. Visual appearance of the coated materials is important for in use situations where the rather dirty look of the high Ag containing films, especially on tiles was rather unattractive. This would be masked if coloured tiles were used but in healthcare situations white tiles are normally used and such colouration would be undesirable. Thus although coatings with higher concentrations of Ag or Cu have higher antimicrobial activity, this has to be balanced with their appearance and durability (see below).

Silica has been used as a physical barrier to prevent or reduce the air oxidation of the metallic copper (Akhavan, 2008). Silica acquires a porous structure and can absorb various ions and organic molecules easily in its pores and on its surfaces. Therefore, silica is expected to be one of the most suitable carriers used for the development of high quality antibacterial and bactericidal materials (Jia *et al*., 2008). Numerous studies have showed that films consisting of silver alone have a low Moh hardness compared to the Moh hardness of silver /silica films (Cook *et al*., 2011).

4.3.1 Durability of coated surfaces

The durability tests show that both samples of glass copper coating (Cu/SiO_2 and Cu/TiO_2) passed the tape test. This suggests that the coating was well adhered to the glass substrate and had abrasion resistance equivalent to steel. This is important in the resistance to wear during the expected lifetime of the products (10–15 y). It is possible that the presence of copper oxide enhances adhesion, as previously shown by (Benjamin and Weaver, 1960). On the other hand, durability and the hardness of the silver coating were dependent on the amount of Ag in the film. Films with a lower Ag content had a Moh hardness of >5, equivalent to steel, and were well adhered to the substrate. However, increasing the amount of silver reduced both the hardness and adhesion. Indeed, the biocide activity of silver films was also affected: films with a higher amount of Ag were more active against

pathogenic bacteria than films with a lower amount of Ag. This may be due to the aggregates formed as results of the interactions between the silver ions with silica which increased when the amount of Ag was increased. Similar findings were also reported in a recent study. The researchers had predicted that there was a negative relationship between the amount of silver, flow rate and the hardness of the film produced. They tested different amounts of silver, ranging from 0.05-1.0 M, as well as different nitrogen flow rates of 0.2, 0.5, 1.0, 1.5 and 2.0. Results showed that the hardest film (4.4) was obtained when a low flow rate (0.2) was used and the Moh hardness decreased by 16%-10% over the range of the silver concentration studied (Cook *et al*., 2011). Moreover, it has been reported that increased silver in the surfaces resulted in a change in surface topography, surfaces features were larger and more rounded (Whitehead *et al*., 2010). In line with these findings, the hardness of copper/silica films produced in this study was due to the use of a low gas flow rate (0.5) and an appropriate concentration of copper (0.25M). Substrate temperature was also reported to affect the activity and the durability of the coated surface. The researchers reported that the optimum temperature for the deposition of TiO_2 coating on samples was 600 ºC and for 250ºC for TiO_2/Ag samples. At a higher temperatures, Ag particles were combined together (analysed using SEM), leading to a decrease of surface area and thus decreasing their killing ability (Le *et al*., 2011). High temperatures enabled some silver to diffuse to the surface (Foster *et al*., 2012). Results of this study also showed that the copper-coated tiles were less hard than the copper-coated glass. This may be due to the glazing on the surface of the tiles. However, the coated tiles showed a good antimicrobial activity when tested against the standard test strain of *E. coli* (Figure 35).

4.3.2 The effects of washing on antimicrobial activity of Cu/SiO_2 coating.

The results showed that the coating (copper-coated tiles) still had antimicrobial activity even after the removal of approximately 75% of the initial Cu concentrations by extensive washing. The variation in antimicrobial activity results could be due to a local variation of Cu in tiles. Also, the wash resistance of the samples was quite different and was dependent on the amount of Cu in the samples. The samples with a low concentration of Cu had a higher washing resistance than the ones with a higher concentration. For this, using an abrasive sponge was easier in controlling the Cu removal than a soft sponge. However, the extensive washing using abrasive sponges was extreme; normal cleaning with a less abrasive cloth will not remove as much copper. This indicates that this kind of coating is durable and should remain active in real use situations for prolonged periods of time and under different environmental conditions. Previous research shows that a thin film of TiO_2 over silver showed scratch resistance and durability higher than films of silver over TiO_2 (Brook *et al*., 2007b). Samples were repeatedly used for the biocidal test and were followed by the stearic acid test to measure the reduction of photoactivity and the samples were cleaned each time. The results showed no measurable reduction in photoactivity over three test cycles. However, in the case of silver over TiO_2 films, some of the silver was removed from the surfaces, hence the surfaces still showed antimicrobial activity (Brook *et al*., 2007b). A recent study showed that washing and recycling (up to eight cycles) of the TiO_2/Cu coated surface affects its antibacterial activity and the activity decreased as the recycling increased, since samples were thoroughly washed after each recycling experiment (Baghriche *et al*., 2012). The decrease in activity was due to a lower release of Cu ions, which decreased to 5-6 ppb/cm^2. However, this small amount of Cu is not considered to be cytotoxic but highly bactericidal, since Cu ions bind to negative groups of the bacterial cell wall and this would facilitate it entering the bacterial cytoplasm. The release of Ti ions was also lowered from 8pbb/cm^2 to 4ppb, which is consistent with the high stability of TiO_2 (Baghriche *et al*., 2012). In

another study, silver ions implanted on stainless steel were tested to demonstrate long-term antibacterial function. Two groups of silver surfaces were used: one group was immersed in tap water and the other was exposed to the air in a dryer. Results showed that the antimicrobial activity of silver ions implanted on stainless steel remained good even after a year of exposure to both air and water (Chen *et al*., 2013). The resistance of the coatings to cleaning shown here indicates that the activity can be retained and thus the surfaces may be useful in real life in helping to overcome bacterial infection, especially in healthcare sectors.

4.3.3 The effect of different temperature on antimicrobial activity of copper-based coatings

Bacteria suffering from different stressful environmental conditions mostly go through a physiological differentiation stage known as the viable but non-culturable state (VBNC). In this state, VBNC bacteria are still viable and continue their metabolic activity and respiration. However, they cannot be detected as CFU by the plate-counts method because they do not multiply (Oliver, 2000). Bacteria, in VBNC state are not able to cause infection but as soon as it resuscitated, they retain their toxic ability (virulence). Therefore, VBNC state should be considered when evaluating bacterial toxicity (Oliver, 2005). It has been reported that copper (Grey and Steck, 2001), and silver (Jung *et al*., 2008), induce the bacteria to enter the VBNC state. In contrast, a recent study showed that bacteria exposed to copper surfaces did not enter the VBNC state; rather, they were completely killed (Santo *et al*., 2008). This was in good agreement with the present study; since the cells that were exposed to Cu-SiO_2 appeared mostly red (Live/dead staining) which indicates that the membrane was damaged in case of *E. coli* and *S. aureus*, and there were also fragments of DNA (in the comet assay) in case of *E. coli*. Even though the results reported in this study and in Santo *et al.'s* 2008 paper contradict those by Jung *et al.* (2008), this may due to the fact that resuscitation of the bacteria after having been exposed to copper was not attempted. However, membrane (seen in both

this study and Santo) and DNA damage suggests that the cells were dead and not in the VNBC state so resuscitation of bacteria would not be appropriate.

Physical environmental conditions such as low temperatures (4°C or 6°C) are known to increase the survival of most types of pathogenic bacteria, including MRSA and *E. coli* (Kramer *et al*., 2006). Despite this, in this study the survival periods of ESBL^{+}*E. coli* and *A. baumannii* were decreased when they were exposed to the CuO-TiO_2 surface at 5°C. In fact, the reduction in activity of both strains was greater when UVA light was used. Cell killing probably occurs as a result of the accumulation of damage to the wall/membrane until the membrane integrity is impaired. The reduced activity may be due to the slower production of ROS at 5°C, resulting in slower membrane damage. In contrast, Cu/SiO_2 was highly active at higher temperatures (35°C) against pathogenic MRSA 15 and Standard test strains *E. coli* and *S. aureus*. However, at this temperature there was reduced survival of bacteria on control surfaces, which may have been due to an increased release of SiO_2 ions as effect of temperature. It has been shown that the SiO_2 nanoparticles (NPs) able to kill different types of bacteria at 25 $^{\circ}$C (room temperature). The SiO_2 NPs are more active as biocide against *E. coli* with 58% killing than aluminium oxide (Al_2O_3) (36%), but Al_2O_3 NPs was more active against *B. subtilis* and both surfaces (SiO_2 and Al_2O_3) were equally active against *Pseudomonas fluorescens* (Jiang *et al*., 2009). Therefore, room temperature was used for the rest of the tests in this study. The differences observed in the killing rate with the temperature may be due to an increase in the release of copper ions or to an increase in the copper intake by the bacterial cell, with a higher temperature. The opposite effect may occur in cases of low temperature. In this study, it is noted that at room temperature, most bacteria strains that showed high sensitivity to being killed by copper were killed through the biphasic effect. A quick phase caused a 1-2 log reduction in viability within the first 1 to 2 h of exposure. In a second phase, all remaining live cells were inactivated within 4 to 6 h. Similar findings were reported by Faúndez *et al*. (2004). They showed

that the antimicrobial effect of copper against *Salmonella enterica* and *Campylobacter jejuni*, which are known to cause food borne diseases, was higher at 25°C compared to the activity at 10°C with only a 2 log reduction after 8 h compared to a 4 log reduction after 4 h at 25°C for both strains. The killing rate of *E. coli* O157 on copper (dry method) at 5.5°C was lower than the killing at 23°C (Santo *et al*., 2008). Another study showed the complete killing of different MRSA strains (MRSA, EMRSA-1 and EMRSA-16) on copper surfaces compared to stainless steel at 4°C but with time differences: 6 h was required for complete killing at 4°C, whereas only 45, 60 and 90 min were required at room temperature for the strains, respectively (Noyce *et al*., 2006). However, a contradicting result has been reported in a recent study. This study showed that there was no effect of copper on the survival time of MRSA and *P. aeruginosa* at 4°C, whereas *K. pneumoniae* and *A. baumannii* were killed at 4°C but with a longer time compared to their killing time at room temperature (Mehtar *et al*., 2008). The different results reported in the above studies on the killing of MRSA strains on copper surfaces may be due to two points: different MRSA strains were used, since different strains respond differently to copper, as we have shown in this study; and a different inoculated method (dry or wet), because it had been repeatedly reported that the killing of copper on the dry method is more effective than using wet methods (Santo *et al*., 2008). It has been reported that the efficacy of copper surfaces continued even at a lower level of humidity which typically found in the hospitals. Researchers demonstrated that copper alloys gave a > 5 log reduction under different levels of humidity and temperatures (~90% RH-35°C, ~20%RH-35°C and ~24 % RH-20°C) compared to silver containing materials, which showed no reduction at ~20 % RH and 35°C (similar to hospital environments) (Michels *et al*., 2009). These results indicate that the efficacy of copper as an antimicrobial agent is retained in varying environmental humidity.

Overall, better antimicrobial activity was noted for all copper-coated surfaces at room temperature than at 5°C. The results suggest that, even though there is reduced activity at lower temperatures, the

coatings may have applications in reducing surface bacterial contamination in refrigerated areas, for example, those used for food storage. However, despite the fact that the continuous copper ion release is essential for the efficacy of copper surfaces, copper ions may affect the foodstuffs themselves. Therefore, before application of the copper and the new generation of copper alloy surfaces in food processing areas, advanced studies have to be conducted to determine if the copper ion release is necessary for copper efficacy. In fact, serious consideration has to be given to which surfaces in food industry and preparation would be best suited for copper biocidal surfaces (Warnes *et al*., 2012).

4.3.4 Killing mechanism of copper

Copper is able to generate hydroxyl radicals even in the dark. These radicals are powerful non-selective oxidants that are able to cause damage to the biomolecules by carboxylation of proteins, membrane peroxidation and DNA damage by base modification (Warnes *et al*., 2012). However, the exact mechanisms by which copper ions kill bacterial cells are still under debate. It has been reported in several studies that membrane damage is a key initial event in contact killing on a copper surface. This was demonstrated for several organisms and by using different techniques, including direct microscopic examination of cells using different dyes such as 5-cyano-2,3-ditolyl tetrazolium chloride or rhodamine 123 staining of respiring cells (to differentiate live metabolically active from dead metabolic inactive cells), or by the live/dead staining technique (propidium iodide and Syto9). However, the exact mechanisms that explain how membrane damage occurs and how outer and/or inner membranes of bacteria are affected are still not clear (Mathews *et al*., 2013).

Copper has the ability to bind to DNA bases, causing double and single strand breaks. This is due to free ions and hydrogen peroxide, which results through the Fenton reaction (to give ROS). However, a study by Macomber *et al*. (2007) found that the toxicity of copper ions does not come from DNA

damaging free radical reactions. They suggested that compartmentalisation of the hydroxyl radicals occurs in the periplasm of the cell. Following this, the toxicity of copper may be due to damage of the metalloenzymes by ROS. The effects of hydroxyl-radical damage is short range as the radicals react very quickly; therefore, damage to the DNA cannot occur if the sites of hydroxyl-radical generation are separated from the nucleic acid site (Macomber *et al*., 2007). The hypothesis that the killing action of copper does not primarily originate from the targeting of DNA was also reported in another study. Research showed that the killing of *E. coli* (Gram-negative) on metallic dry copper surfaces was due initially to membrane damage caused by copper ions, and the finding was based on the fact that all copper-demanding enzymatic reactions in *E. coli* occur in the periplasm or at the cytoplasmic membrane and there is no known cytoplasmic enzyme that uses copper as a cofactor (Santo *et al*., 2008). On the other hand, Warnes *et al*. (2010) have shown that DNA was rapidly destroyed in enterococci (Gram-positive bacteria) when exposed to copper surfaces using a genomic DNA fragmentation assay and SYTO 9 staining and suggested that this was not due to hydroxyl radicals but was due to superoxide and copper ions. They suggest that the inhibition of respiration with damage to the integrity of the cell membrane may facilitate the access of copper ions and generated ROS to the DNA, directly causing rapid damage. A similar finding was reported in another study using the same staining method: researchers demonstrated that killing of MRSA on copper surfaces was due to the loss of DNA integrity, respiration and had little or no effect on cell membrane integrity (Weaver *et al*., 2010). However, respiration is a process that occurs on the cytoplasmic membrane and it depends on intact membranes for build-up and use of a proton-motive force across the membrane for ATP production. Therefore, it is hard to believe that damage occurs only in respiratory proteins set in the membrane but not in the membrane itself (Santo *et al*., 2012). Additionally, in a recent study, the effect of copper on *E. coli* (K12) proteins was studied. The abundance of *E. coli* proteins involved in secondary metabolite biosynthesis, transport and

catabolism, including efflux proteins and multidrug resistance proteins, were increased after contact with copper, whereas no up-regulated proteins that belonged to DNA replication, recombination and repair were identified on the *E. coli* exposed to the copper surface, except the DnaX protein involved in DNA replication but not specifically in DNA repair (Nandakumar *et al*., 2011). In line with these observations, the results in the present study showed two different aspects: the integrity of the cell membrane of both types of bacteria (Gram positive and Gram negative) were affected by copper using fluorescent staining (SYTO 9), which binds to intracellular DNA as a measure of cell permeability, but with time differences, and Gram negative was more sensitive than Gram-positive. The damage of DNA occurred only on Gram-negative bacteria but not on Gram- positive bacteria. In contrast, Santo *et al*. (2011) demonstrated that the death of *E. coli*, and *Deinococcus radiodurans* on wet or dry copper surfaces was due to membrane damage only and genotoxicity did not occur. *D. radiodurans* has a variety of efficient DNA repair functions and is able to re-join the DNA fragment into complete genomes over a period of 3 to 4 h. After repair, cell division begins as normal. Therefore, if contact with metallic copper caused DNA damage in cells, then *D. radiodurans* would be expected to recover from this stress. However, the opposite action was observed. *D. radiodurans* cells were as sensitive to killing on dry copper as *E. coli* cells, and their killing sensitivity was even higher when exposed to moist copper (Santo *et al*., 2011). It has been reported that fluorescent dyes such as *Bac*Light and SYBER Gold lose their florescence when performed directly on metallic copper and therefore removal of cells from copper surfaces before staining was recommended (Santo *et al*., 2011, Santo *et al*., 2012). However, the negative staining artifacts may explain the contradictory results reported in Weaver *et al*. (2010) and Santo *et al*. (2012). Warnes and Keevil (2011) reported contradicting results using a different staining method. They showed that the cell membrane of enterococci is compromised on a dry copper surface only after the cells are dead and in their recent study they predicted that immediate cytoplasmic membrane depolarisation occurs on *E.*

coli O157. To ensure that these results were not due to interaction between copper and the dye, bacteria *(E. coli* O157) were removed from coupons at specified time points and stained using lipophilic dye rhodamine 123 (Rh123), which accumulates on the inner membrane of intact cells. Moreover, the peptidoglycan (PG) of *E. coli* cells exposed to copper, followed by lysozyme (which break the β (1- 4) linkage between *N*-acetyl glucosamine and *N*-acetyl muramic acid in the peptidoglycan bacterial cell wall) treatment to loosen the cell wall was visualised using Gram-staining. Results showed that cells aggregated into large clumps and retained the crystal violet-iodine complex, which is an indicator of PG damage and this effect did not occur immediately on contact, suggesting that cytoplasmic membrane depolarisation occurs first. Therefore, the outer membrane and their structures in Gram- negative bacteria is the target toxicity for the copper surface since these are the first parts of the cell that come into contact with the copper surface (Warnes *et al*., 2012). Furthermore, the DNA degradation of *E. coli* O157 on a dry copper surface was also reported, but with a longer time compared to enterococci. The delayed degradation on *E. coli* compared to enterococci may be due to the protection of the nucleic acids by the periplasmic space and the extensive cell aggregation after being exposed to copper (Warnes *et al*., 2012). This was in complete contrast with results reported by Santo *et al*. (2012). However, different diagnostic methods were used; in Santo *et al*. (2012), their method was based on detecting small changes in the DNA using PCR and mutagenicity assays and in Warnes *et al*. (2010) and (2012), wet and dry methods were tested and purified DNA of whole cells and purified plasmid DNA was used.

It is worth noting that the staining techniques used in the above studies such as live/dead and respiration indicator staining are functions to differentiate between live and lethal damage dead cells and they do not make the connection to the possibility of membrane damage as the underlying mode of action. Therefore, this is indirect evidence as these methods (staining methods) measure cell activity but does not specifically indicate membrane damage (Santo *et al*., 2011).

Enumeration of viable and none viable bacteria cells can be performed either by direct method plate count or by using fluorescence microscopy and digital image which takes only 15 min. However, the results will show at least one log (CFU/cm^2) higher than the plate count method. This may due to two different factors; because bacteria do not grow under the conditions provided in the plate count method and/or large numbers of bacteria remain on the surfaces after swabbing (Fuster-Valls *et al*., 2008). Different results have been reported by Khan *et al*. (2010). They showed that the use of flow cytometry (a rapid and accurate method which able to enumerate thousands of cells with intact membranes within a few seconds) in combination with staining techniques such as live/dead staining give an accurate and precise cell count results compared by other cell count techniques such as SYTO 9, which is supposed to indicate live cells with intact membranes. However, SYTO 9 can sometimes be preferentially excluded from some bacteria. They also show that different staining gives different cell count results for different bacteria species. Dye selection for each bacterial species is recommended to achieve accurate cell counts (Khan *et al*., 2010). This indicates that using staining to enumerate bacterial cells is not the right method. Therefore, in this study SYTO 9 was used only as membrane damage indicator.

Recent studies on the molecular toxicity of copper showed that bacterial membrane damage is the first step in contact killing by copper. The findings were based on identifying the damaged protein or lipid bound membrane as the sensitive targets in cells. A few bacterial cells exposed to the copper surface were selected to map the distribution of bacterial composition compared to the healthy bacterial cell. The results showed the loss of protein in the bacterial cell after contact with copper coating and damage to these specific targets led to the rapid and efficient bacterial killing process (Wei *et al*., 2014).

Even though the toxic effect of copper ions on bacteria cells remains unclear, the penetration of copper ions into the cytoplasm is the main factor in contact killing is non-debatable and is supported by several observations. First, cells pre-adapted to ionic copper and thus equipped with regulated copper resistance mechanisms are more resistant to contact killing than bacteria with deficiencies in their copper resistance system. Second, large amounts of copper entered the cytoplasm in different bacterial species. Finally, copper chelators like EDTA significantly inhibit or even prevent contact killing (Mathews *et al*., 2013). So far, only one or two types of bacteria strains were used in different studies; therefore, these findings for copper mechanisms are not final and extended studies are needed.

Chapter 5

The activity of coated surfaces *in situ* use

5.1 Introduction

Microbes have a natural ability to survive and colonize commonly-touched surfaces in health care facilities. The routine and terminal cleaning of surfaces using a hospital-grade disinfectant can effectively remove microbes from surfaces, but studies have shown that surfaces are not terminally cleaned, and may become re-contaminated within a short time (a matter of minutes) (Schmidt *et al*., 2013). Moreover, the traditional routine cleaning methods reduces both microbial growth counts and organic soil, but the effect is not large, since many sites have similar cfu values after cleaning as they did before. In fact, some indicator organisms such as MRSA and MSSA are not necessarily removed by routine cleaning of near-patient hand-touch sites (high risk sites) (Mulvey *et al*., 2011). It has been reported in a recent study that there was no difference in cleaning effectiveness between a traditional cleaning method (quaternary ammonium disinfectant applied with a wetted cloth) and the PureMist system (the disinfectant applied in the form of a microdroplet and allowed to dry in place and does not require mechanical abrasion or the removal of residual material), with a mean reduction of microbial burden (MB) of 84% for the traditional method versus 88% for the Pure Mist method (Schmidt *et al*., 2012a). In healthcare situations, surfaces are usually made of stainless steel due to its clean look, and because it is stable and inert under most circumstances. It has been suggested that metals with antibacterial properties such as copper might be helpful in overcoming surface contamination, especially in a healthcare environment where HCAIs cause severe problems (Airey and Verran, 2007). A recent study has reported that several human pathogenic bacteria die in one to two hours when they come in contact with dry copper and copper alloy surfaces at room temperature.

In contrast, almost no reduction was seen on stainless steel after several hours and even days (Michels *et al*., 2008). In 1983, an experiment was conducted by Kuhn who assessed the growth rates of streptococci and staphylococci on stainless steel and brass. Kuhn showed that there was a heavy growth on stainless steel doorknobs compared to a sparse growth on brass doorknobs. Kuhn recommended that hospitals should retain and maintain their old brass material (Kuhn, 1983). Despite this, although there has been extensive laboratory tests on the antimicrobial properties of copper and copper alloys, there have been relatively few studies showing a reduction of contamination in real use situations, and the majority of studies have concentrated on highly touched surfaces.

In this study, the antimicrobial properties of coated surfaces were tested according to the BS ISO standard method (Anon, 2011) which allows determination of the relative activity of different coatings under standard conditions and against different organisms. However, the method in which a thin film of bacterial suspension is held in close contact with the coated surface does not reflect the type of contamination that is likely to occur in real use situations. The only real test is to see whether or not the *in vitro* antimicrobial activity is maintained *in situ*. The activity of various coatings therefore tested in an intensively used situation.

The performance of the Ag-SiO_2 coated tiles, Cu-SiO_2 coated tiles and glass and the samples provided by OCAS were investigated *in situ* in a ladies toilet facility and the Cu-SiO_2 coated tiles were also investigated in two hospital sluice rooms. Although the contamination of the samples would be due to chance events it was hoped that this would give information on in use activity with natural contamination. The samples were intend to be left in place for >1 year and sampled at regular intervals to test for the levels of surface contamination. Counts of bacteria on the surfaces were determined by swabbing using the standard protocol (section 2- 6.2) and determining a viable count using dilution and plating. Counts were corrected to cfu cm^{-2}.

5.2 Results

5.2.1 Activity in the ladies toilet facility

5.2.1.1 Antimicrobial activity of Ag/SiO_2 coated tiles and$Cu\text{-}SiO_2$ coated tiles and glass prepared by CVD in a ladies toilet facility at the University of Salford.

Coated tiles Cu/SiO_2 coated glass, and OCAS samples were mounted on a wooden board and placed in the Ladies toilet facility as described in section 2-6.1. The samples were placed at random to try to avoid positional bias and the board was rotated by 180° twice a week. The surface contamination was assessed by swabbing ¼ of the coated surface of the samples. A different ¼ was sampled for each of the first 4 samplings. The board was left in place for a little over 18 months but the study was then terminated, partly because the board had clearly been very wet (Figure 64) and this may have affected the performance of the samples in an unknown and unpredictable manner as it was not possible to find out what had happened to the board. The activities of coated tiles (Cu/SiO_2; Ag/SiO_2 and Cu/SiO_2 coated glass) are shown in Figures 61, 62 and 63. The activity of coated tiles (Cu/SiO_2 and Ag/SiO_2) was better than coated glass, with 98% lower surface contamination than the control tiles after the first 18 weeks on both types of coated tiles and 90% lower on Cu/SiO_2 coated glass. The increase in overall contamination between months 3 and 4 was ascribed to increased use of the toilets following return of the university students after the summer vacation. As the tiles gave such high counts and it was necessary to resample areas of the samples that had already been sampled, the tiles were cleaned by wiping with a detergent solution and allowed to dry then retested after 30-42 weeks. On this occasion only the Cu/SiO_2 and Ag/SiO_2 coated tiles gave a reduction, the Cu/SiO_2 coated glass had higher counts on the test specimens than the controls. The samples were therefore thoroughly cleaned and finally rinsed with sterile water in week 43 to try to remove any residual contamination. This restored activity and samples from weeks 46-64 gave reductions of 83, 78 and 73% for Cu/SiO_2, Ag/SiO_2 and Cu/SiO_2 glass respectively (Figures 61, 62, 63 and Table 9). Cleaning

was repeated on week 65 and the Ag/SiO_2 coated tiles from weeks 68-85 again showed higher counts on the test than the controls but the Cu/SiO_2 coated tiles and glass showed reductions of 68.8% and 22.2% respectively (table 9). Overall mean reductions for all tests were lowest on the Cu/SiO_2 coated glass at 65.9 % and highest in the Cu/SiO_2 tiles at 85.1 % (Table 9). In general, the Cu/SiO_2 coated tiles showed a strong ability to retain its antimicrobial activity after each wash compared to Ag/SiO_2 tiles and Cu/SiO_2 glass; and the cfu was higher on Cu/SiO_2 tiles only two times compared to 5 and 6 times on Cu/SiO_2 glass and Ag/SiO_2 tiles respectively. This occurred in weeks 42 and 79 which may have been due to the effects of cleaning (week 42) and to the water damage occurred on week 68 as shown in figure 64.

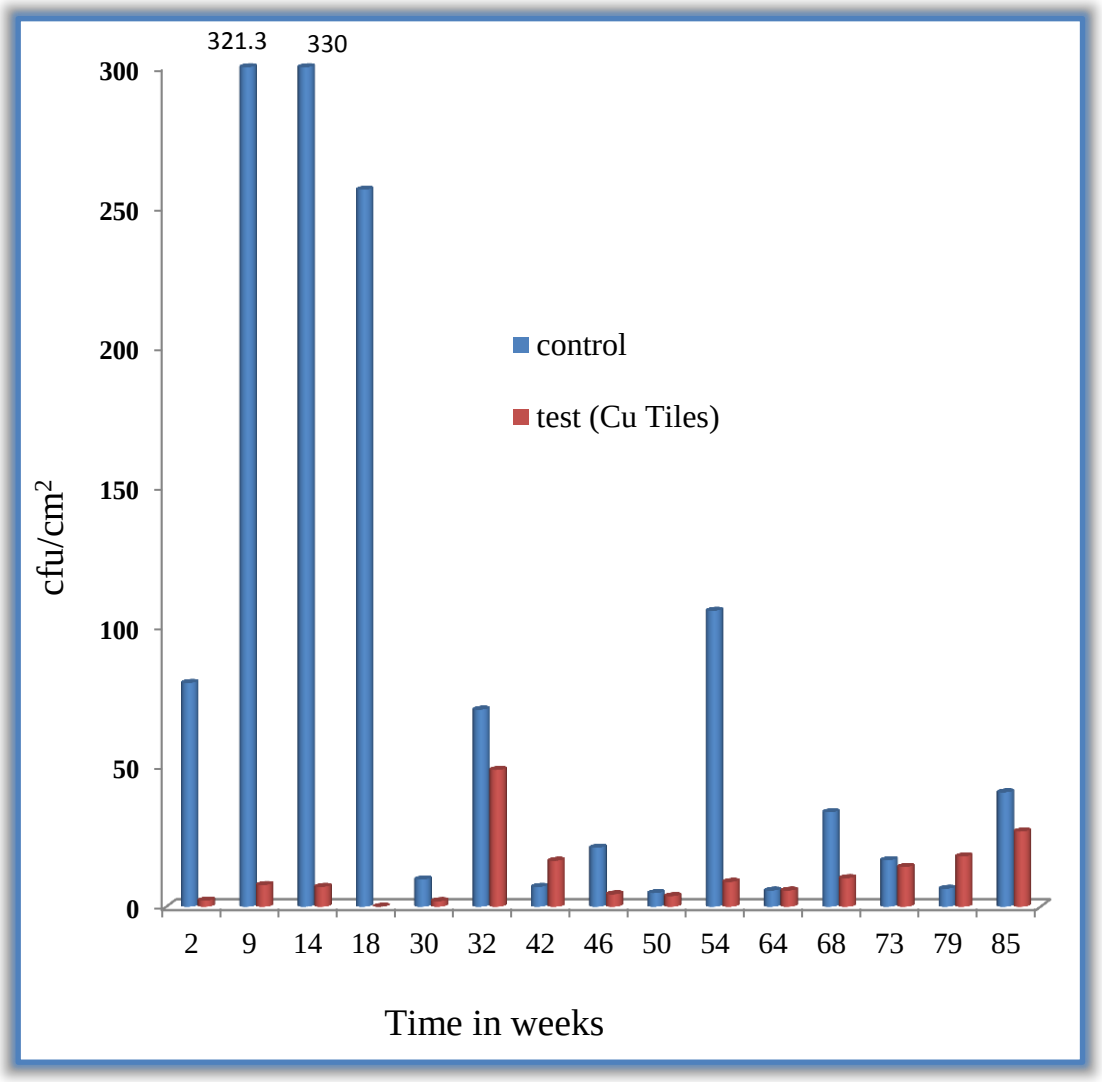

Figure 61 Antimicrobial activities of coated tiles (Cu/SiO_2) test and uncoated tiles (control) in the ladies toilet at Salford University

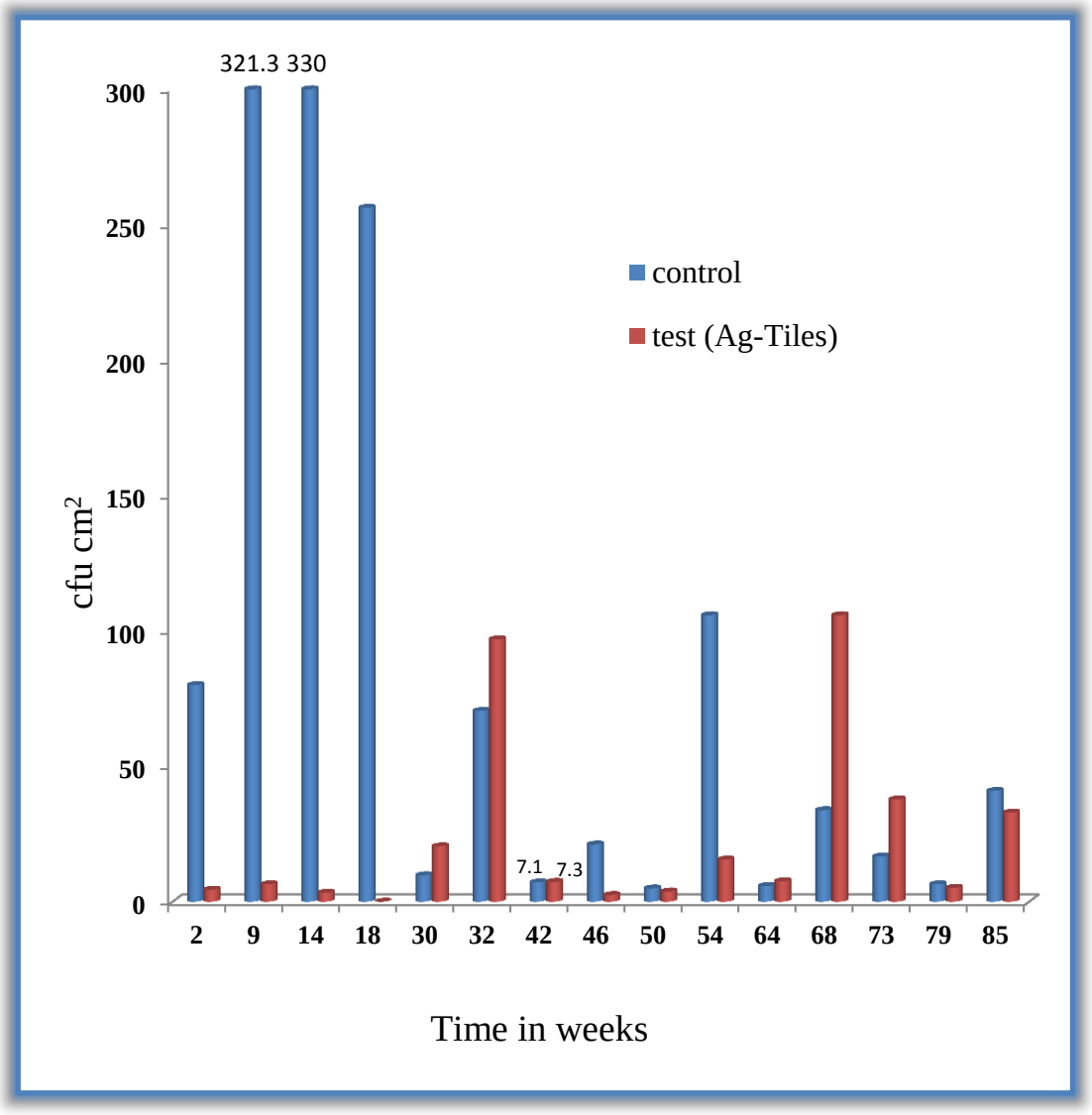

Figure 62 Antimicrobial activities of coated tile (Ag/SiO_2) test and uncoated tile (control) in the ladies toilet at Salford University

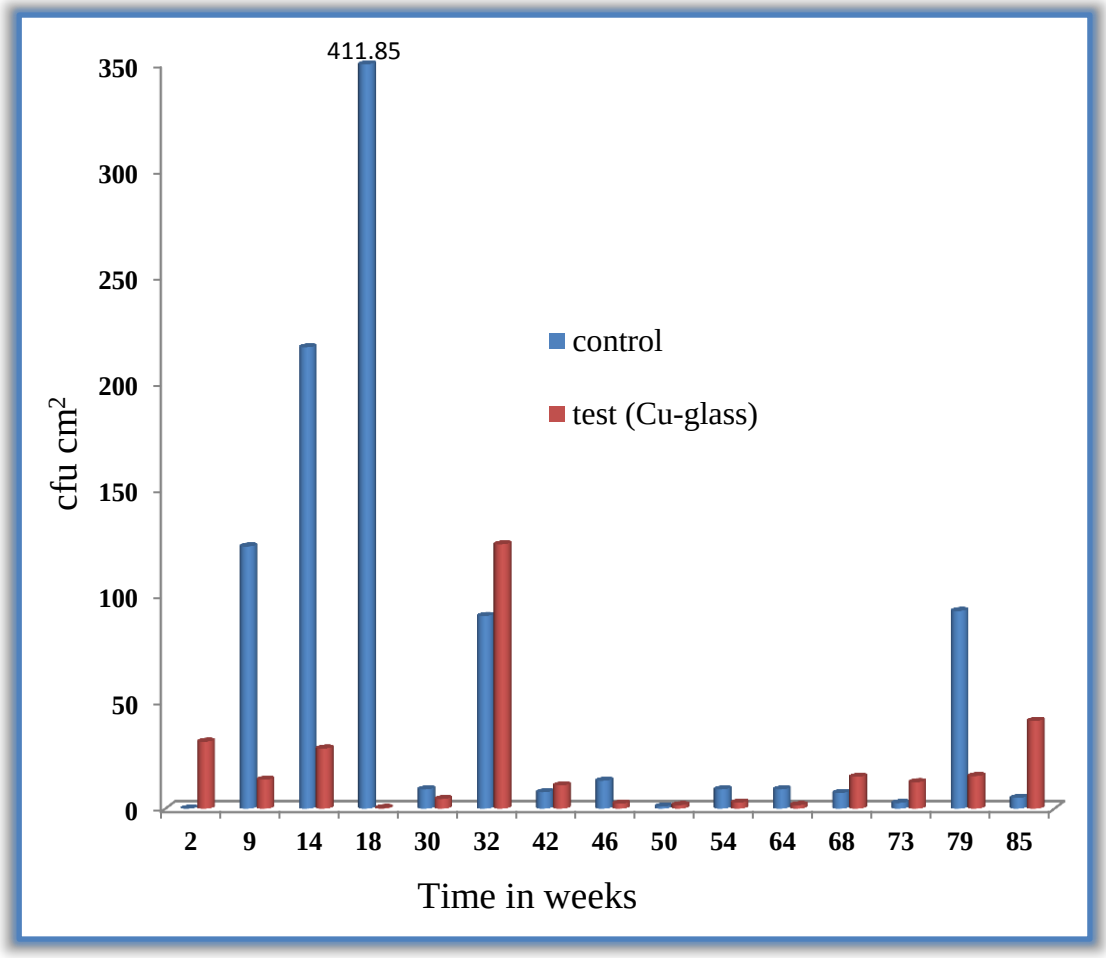

Figure 63 Antimicrobial activities of coated glass (Cu/SiO_2) test and uncoated glass (control) in the ladies toilet at Salford University

Table 9 Summary of reduction in surface count of CVD (Salford University Samples) compared to control surfaces

Reduction in surface count cfu of CVD samples (%)					
Samples	**weeks 2-18**	**weeks 30-42**	**weeks 46-64**	**weeks 68-85**	**Overall mean**
Cu/SiO_2 ceramic tiles	**98.2**	**22**	**83**	**29**	**85.13**
Cu/SiO_2 glass	**90.19**	**0**	**73.4**	**22.2**	**65.9**
SiO_2-Ag ceramic tiles	**98.5**	**0**	**78**	**0**	**70.8**

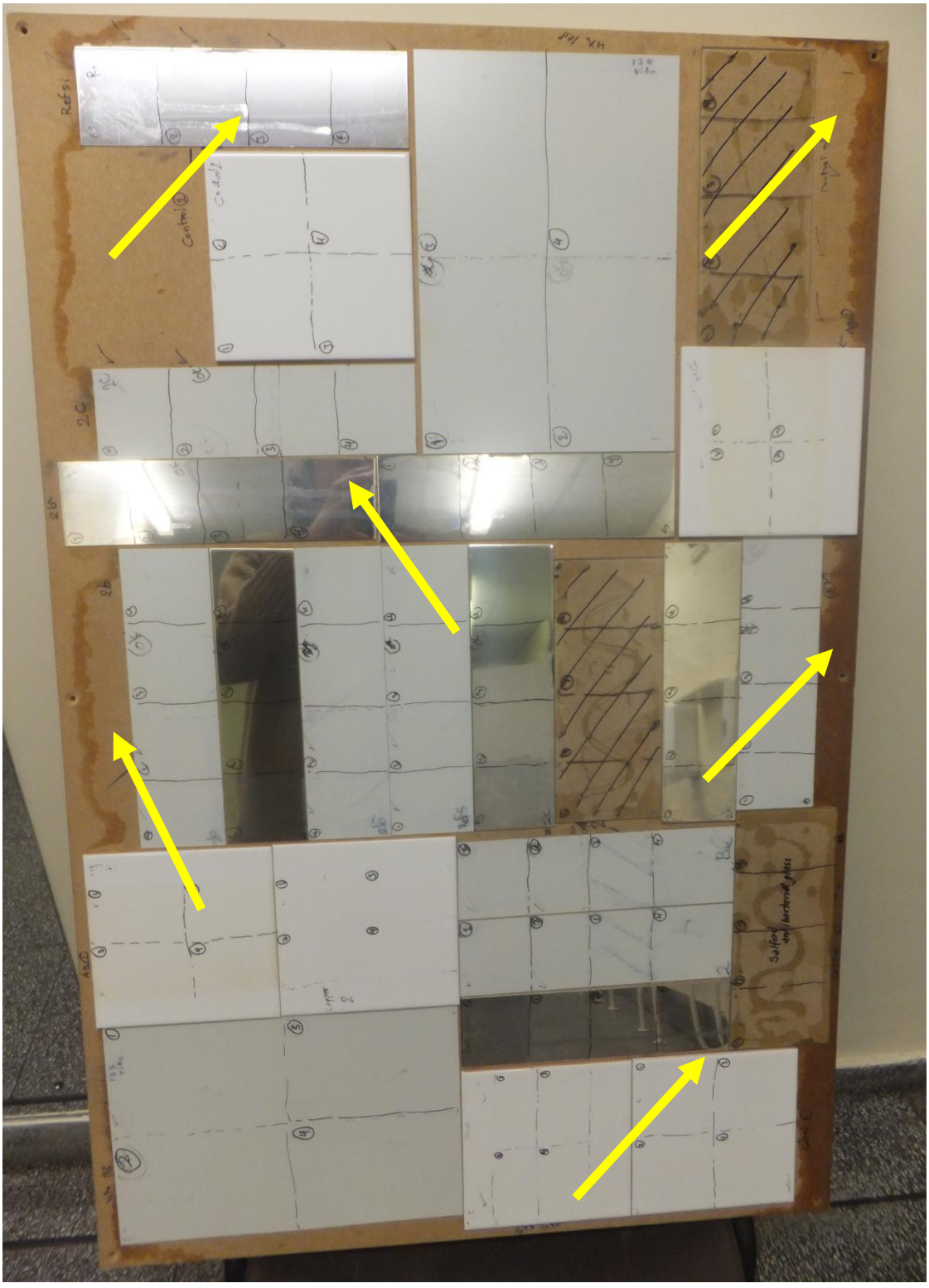

Figure 64 Damage to samples (at week 68) located in Ladies toilet at Salford University (arrows show water marks)

5.2.1.2 Activity of CVD coated steel (OCAS samples)

The results of testing the coated steel and coated painted steel samples are shown in Figures 65 to 70 and Tables 10 and 11. All coated surfaces (steel and painted steel) showed similar results to the CVD coatings (Salford University. samples) in that there was an excellent performance for the first 18 weeks with 92% - 98% reduction in microbial surface count for steel and 91.8 - 97 % reduction for painted steel with counts on the controls reaching over 2000 cfu cm^{-2} on steel and over 3000 cfu cm^{-2} on painted steel (Figures 65 to 70, Tables 10 and 11). After the first cleaning there was a reduction in activity as seen with the CVD coated samples (Salford University. samples). The painted steel coated samples regained activity after the second and third cleanings better than the plain steel and had reductions of 11.9 - 81.9% (Table 11). Most of the steel samples lost activity after third wash (Table 10). Generally after third wash the activity of samples 5 and 2C which had the highest amount of copper were lower compared to the other samples which may have been due to the effect of washing.

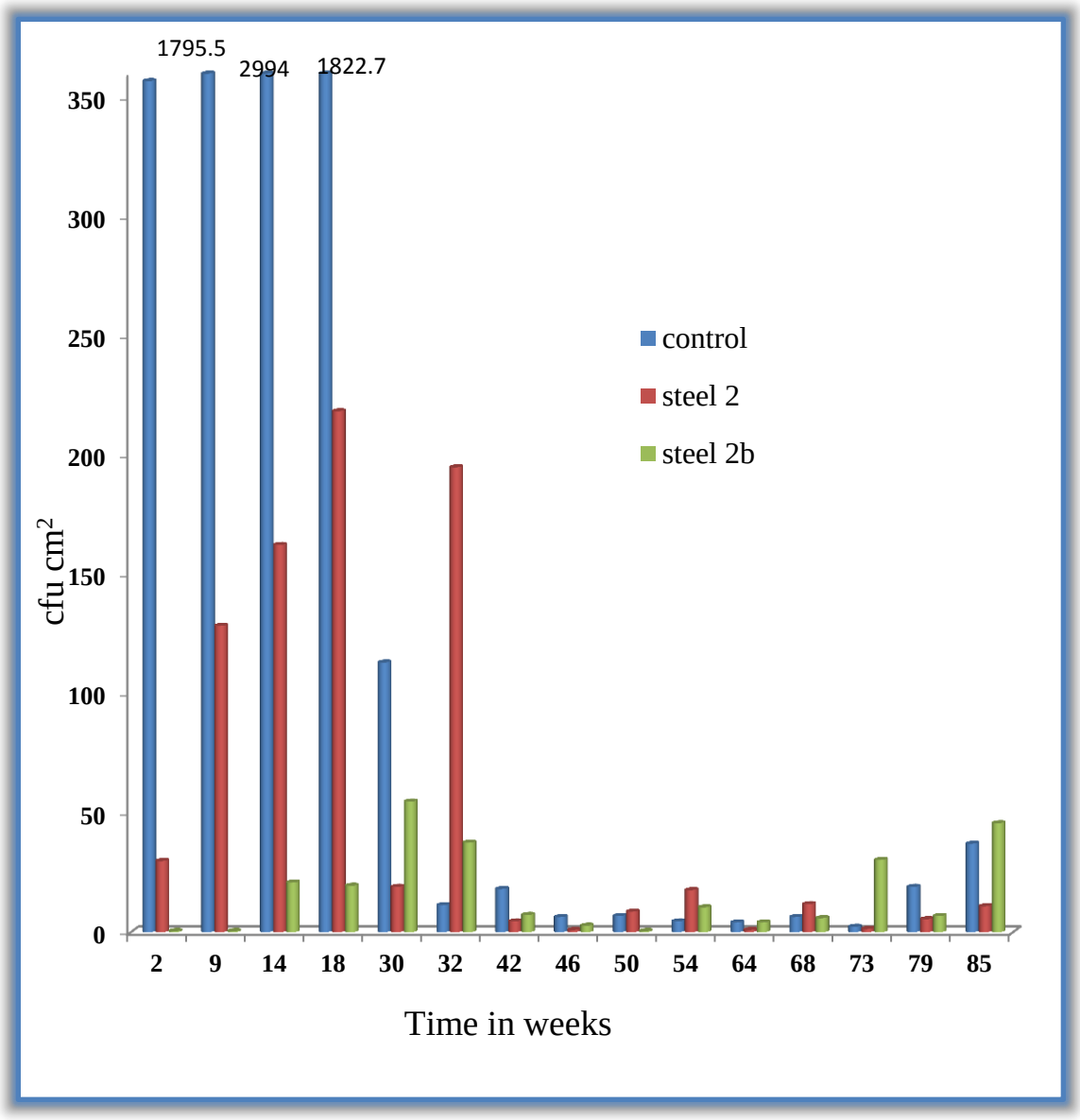

Figure 65 Antimicrobial activities of coated steel tile (2 and 2b- low Cu) test and uncoated steel tile (control) in the ladies toilet at Salford University

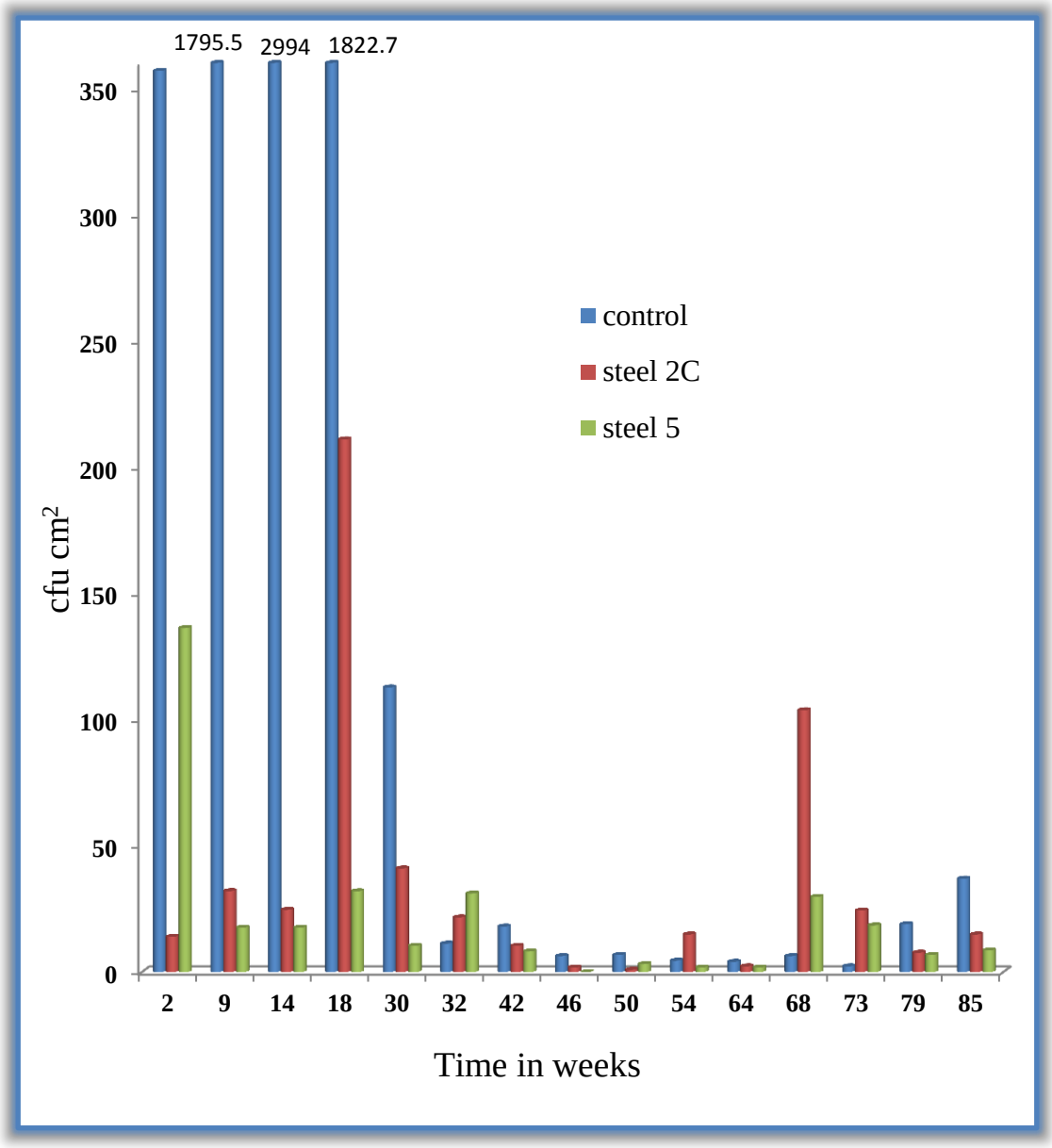

Figure 66 Antimicrobial activities of coated steel tile (2C and 5-high Cu) test and uncoated steel tile (control) in the ladies toilet at Salford University

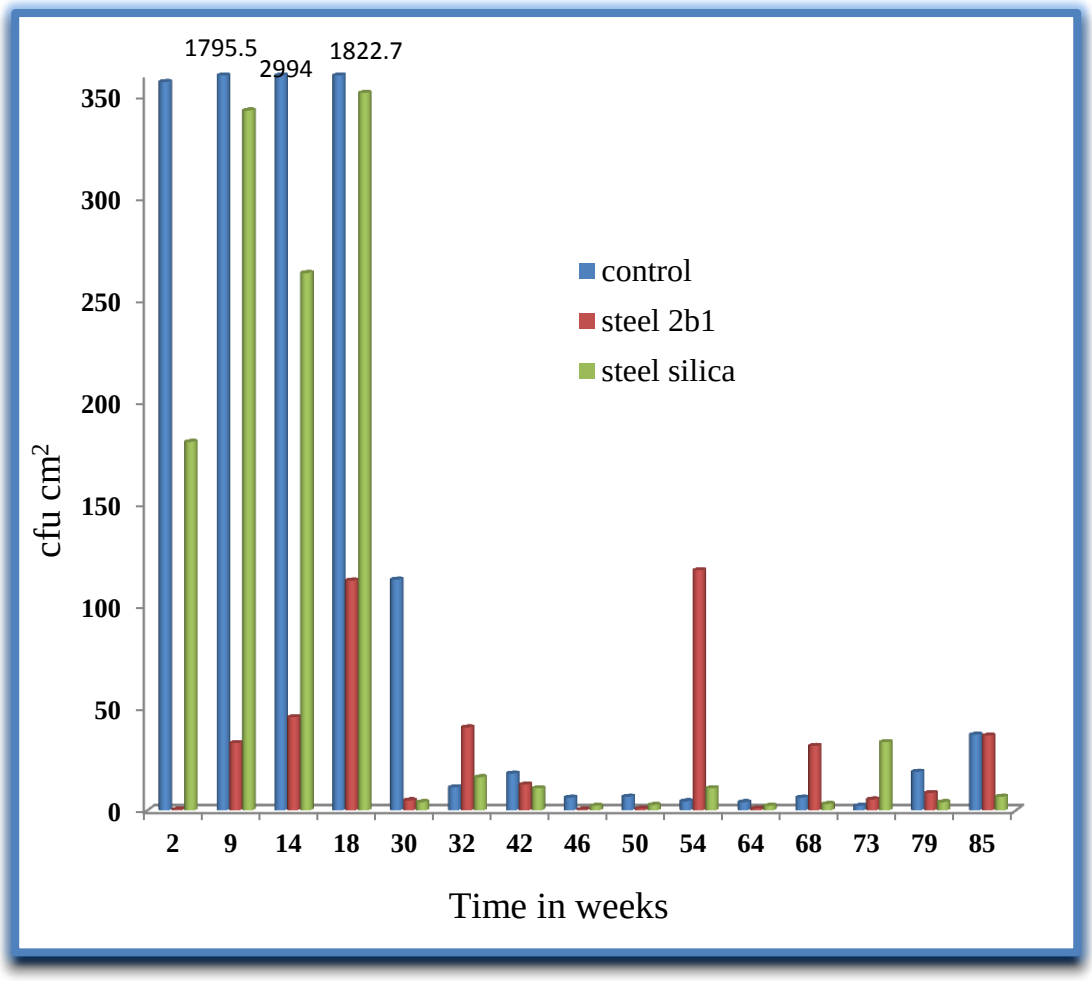

Figure 67 Antimicrobial activities of coated steel tiles (2b 1 low Cu and silica) test and uncoated steel tiles (control) in the ladies toilet at Salford University

Table10 Summary of reduction in surface count of steel samples compared to control surfaces

Reduction in surface count cfu of Steel (OCAS) samples (%)					
Samples	**weeks 2-18**	**weeks 30-42**	**weeks 46-64**	**weeks 68-85**	**Overall reduction**
Steel 2	**92.2**	**0**	**0**	**54.5**	**87.7**
Steel 2b	**98.9**	**30**	**18.7**	**0**	**96.1**
Steel 2b-1	**97**	**59**	**0**	**0**	**93.9**
Steel 2C	**95.9**	**48.5**	**8.33**	**0**	**92.3**
Steel 5	**97**	**65**	**68.7**	**1.39**	**95.2**
Steel Silica	**83.6**	**78**	**16.6**	**26.5**	**82.8**

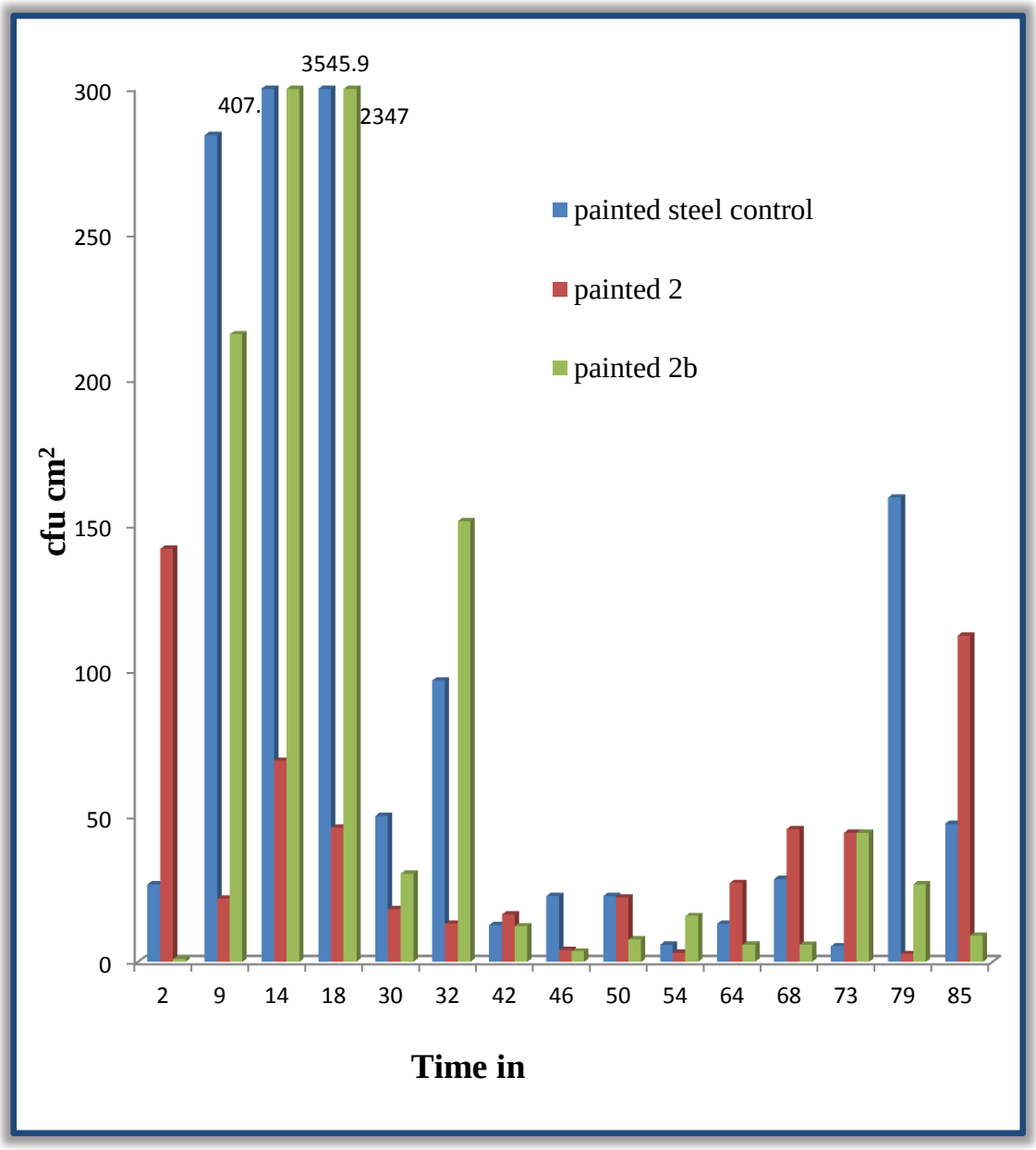

Figure 68 Antimicrobial activities of coated painted steel tile (2 and 2b-low Cu) test and uncoated painted steel tile (control) in the ladies toilet at Salford University

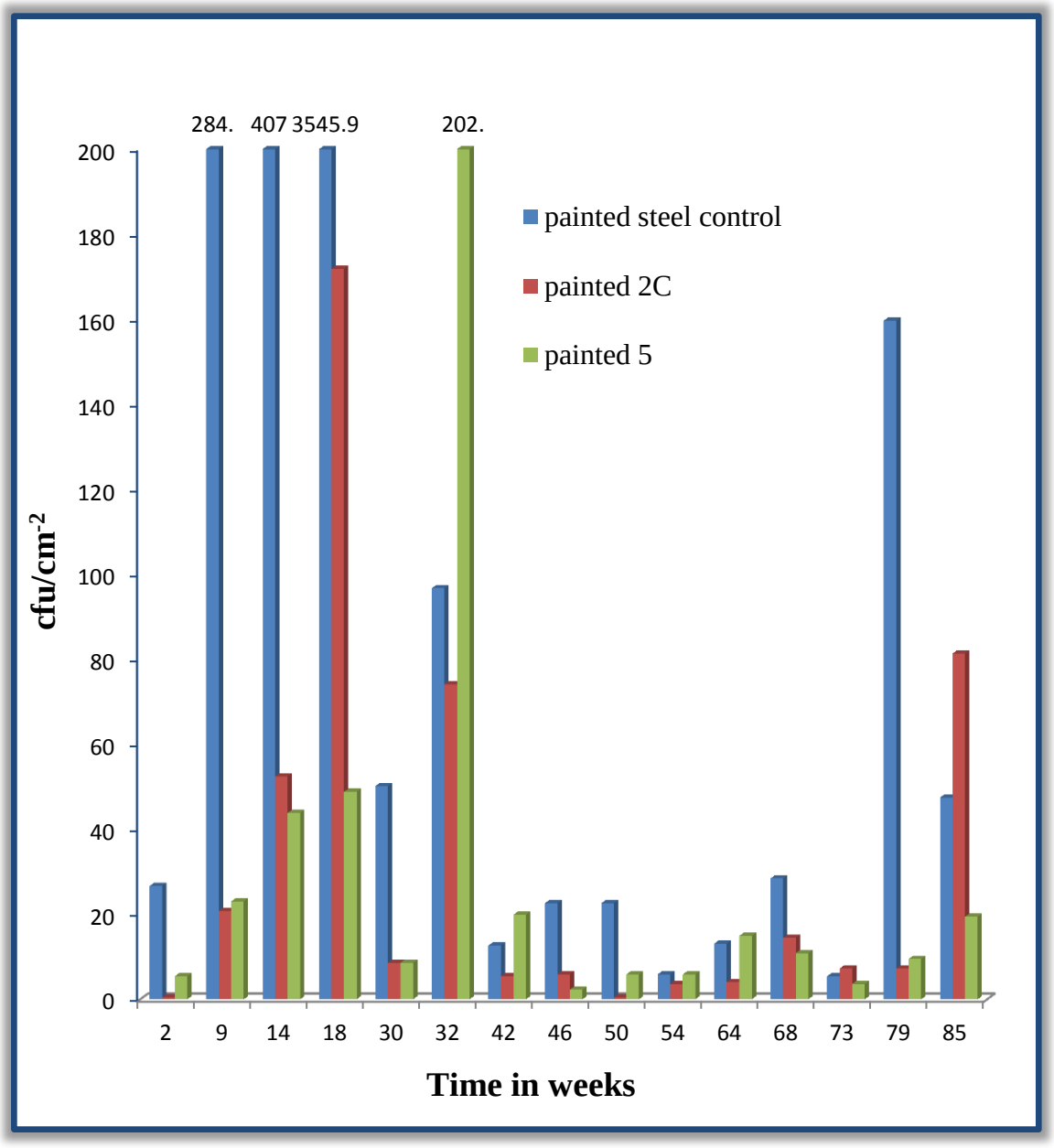

Figure 69 Antimicrobial activities of painted steel coated tiles (2C and 5-high Cu) test and uncoated painted steel tile (control) in the ladies toilet at Salford University

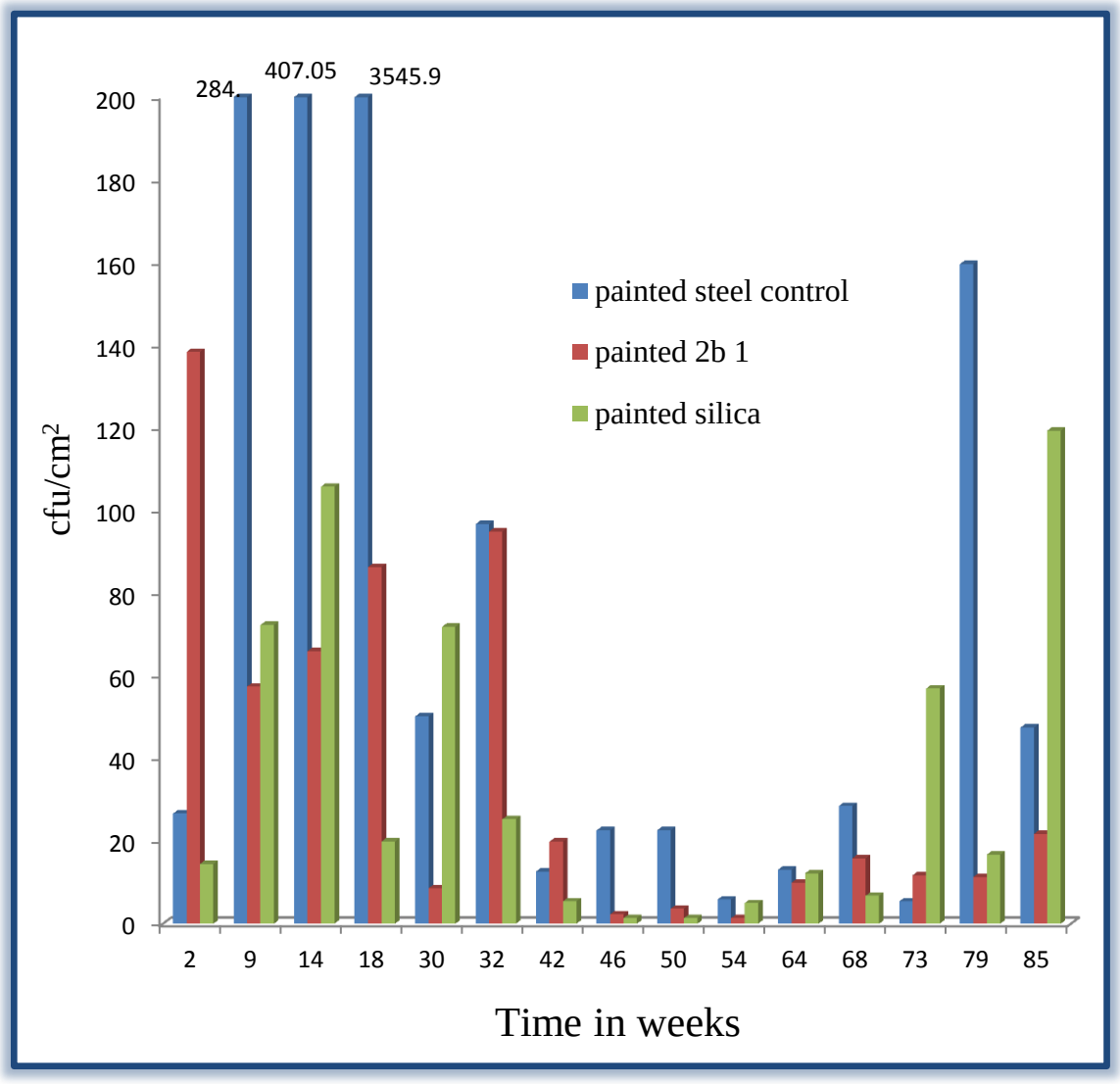

Figure 70 Antimicrobial activities of painted steel coated tiles (2b 1 low Cu and Silica) test and uncoated painted steel tile (control) in the ladies toilet at Salford University

Table 11 Summary of reduction in surface count of Painted steel samples compared to control surfaces

Reduction in surface count cfu of Painted Steel (OCAS) samples (%)					
Samples	**weeks 2-18**	**weeks 30-42**	**weeks 46-64**	**weeks 68-85**	**Overall reduction**
painted 2	**93.4**	**70.2**	**11.9**	**15**	**87.3**
Painted 2b	**91.8**	**22.6**	**73.2**	**74.8**	**87.65**
painted 2b-1	**94**	**44.7**	**78**	**54**	**89.8**
painted 2C	**97**	**0**	**54**	**81.9**	**89.5**
painted 5	**95**	**35**	**69**	**17**	**88.1**
Painted Silica	**30.3**	**0**	**48.5**	**39.7**	**29.9**

5.2.2 Antimicrobial activity of coated (Cu/ SiO_2) and uncoated tiles at Manchester Royal Infirmary (wards 37 and 12)

The activity of the tiles placed in the sluice room in Manchester Royal Infirmary (wards 37 and 12) are shown in Figures (71 a-b to 73 a-c). The coated tiles had 75.8 % lower surface contamination than the control tiles after 20, 36, 44, 72 weeks at site 1 and 68.3 % at site 2 (Tables 12 & 13) on ward 37, and 98 % at site 3 and 85.5 5 % at site 4 on ward 12 (Tables 14 &15). There was no indicator organisms on test tiles on ward 37 compared to the control over the period of testing on site 2. Staphylococci were detected on the coated tile at a much lower level (0.6 cfu cm^{-2}) (Figure 71 b). However, the increased contamination after 44 weeks (at the site 2, Figure 71b) can be ascribed to contamination which occurred in a short time before swabbing. Staphylococci and enterococci were detected on ward 12 at both sites (3 and 4, Figure 73c). However the test surfaces still gave

reductions of 80 -85 % of staphylococci at both sites and 97.85 % reduction of enterococci at site 3 but only 10% at site 4 compared to the control tiles. No coliforms were detected on either test or control tiles.

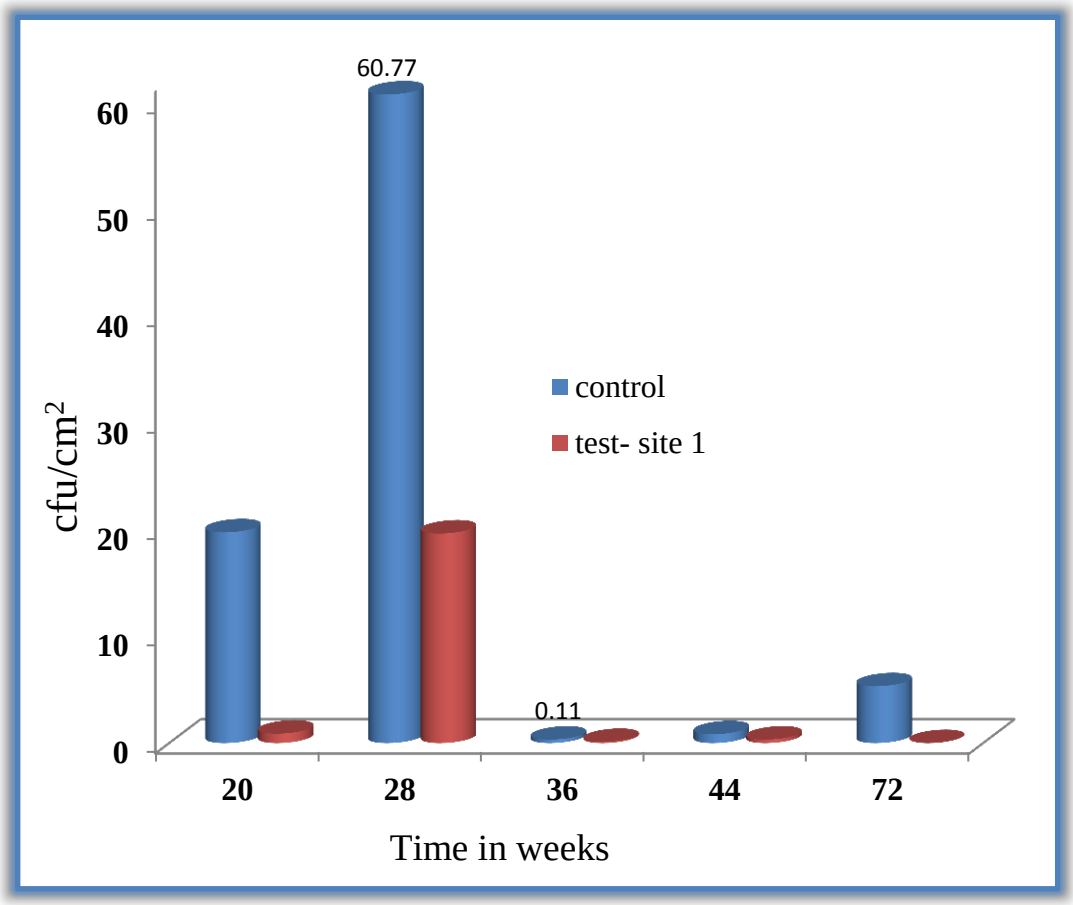

Figure 71a Total viable counts on coated Cu/SiO_2 (test) and uncoated (control) tiles at site 1 at Manchester Royal Infirmary (ward 37)

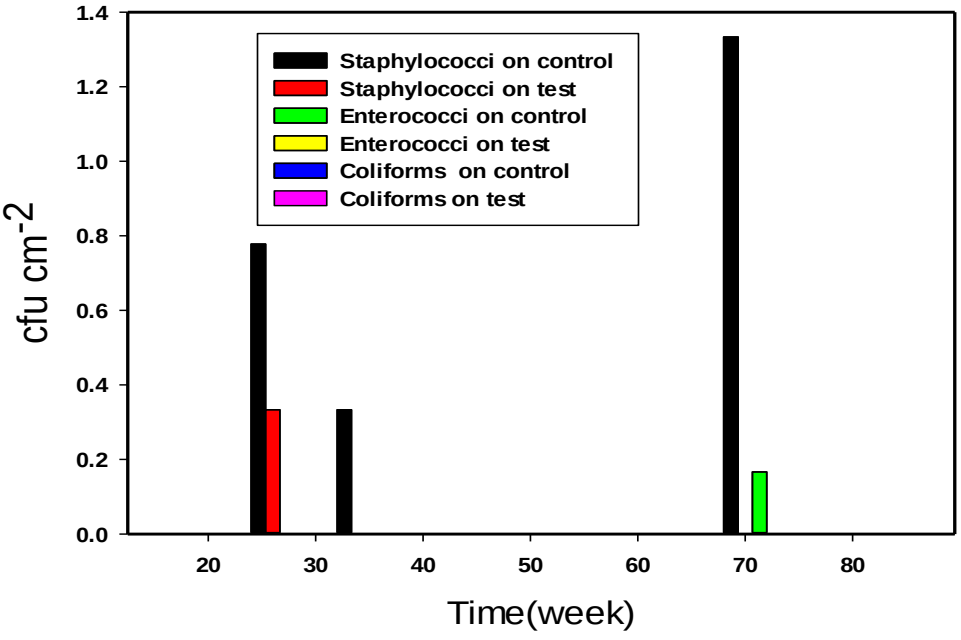

Figure 71b Total viable counts of indicator organisms (Staphylococci growing on Baird Parker Agar, Enterococci growing on Kanamycin Agar and Coliforms growing on Chromocult Coliform Agar) on coated Cu/SiO_2 tiles (test) and uncoated tiles (control) at site 1 at Manchester Royal Infirmary (ward 37)

Table 12 Summary of reduction in surface count of CVD samples (Cu/SiO_2-tiles) compared to control surfaces placed in Manchester Royal Infirmary ward 37 at site 1

Reduction in surface count of CVD samples (Cu/SiO_2) placed at site 1 ward 37 at Manchester Royal Infirmary (%)						
Samples	**week 20**	**week 28**	**week 36**	**week 44**	**Week 72**	**Overall mean**
Total cfu/cm	**95.5**	**67.6**	**99.8**	**60**	**100**	**75.8**
Staphylococci	**-**	**57.1**	**100**	**-**	**100**	**86.3**
Enterococci	**-**	**-**	**-**	**-**	**100**	**100**
Coliforms	**-**	**-**	**-**	**-**	**-**	**-**

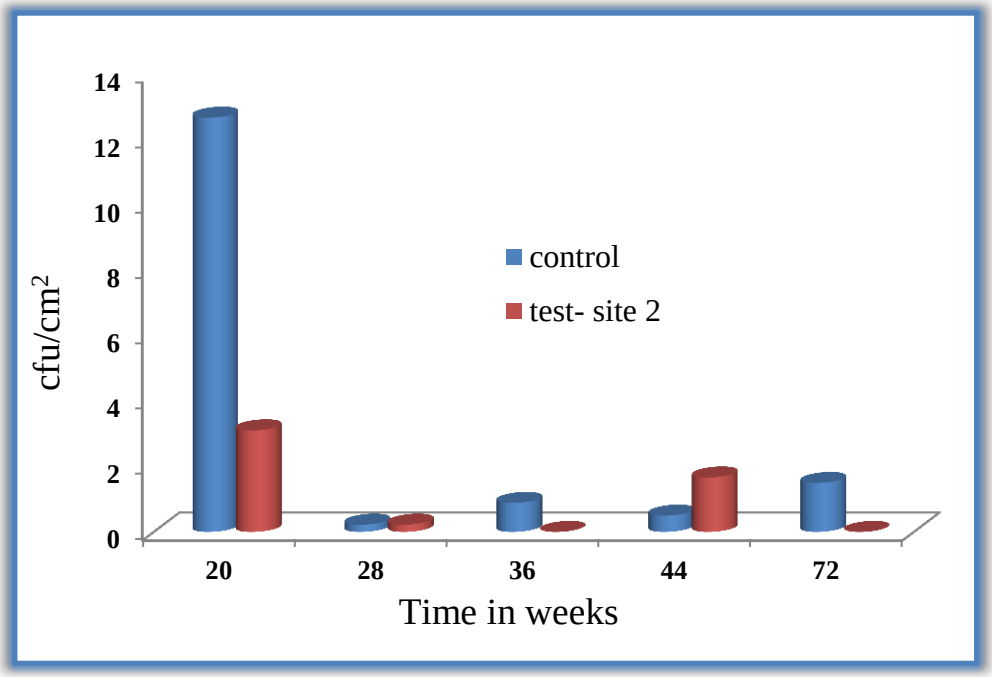

Figure 72 a Total viable counts on coated Cu/SiO_2 tiles (test) and uncoated tiles (control) at site 2 at Manchester Royal Infirmary (ward 37)

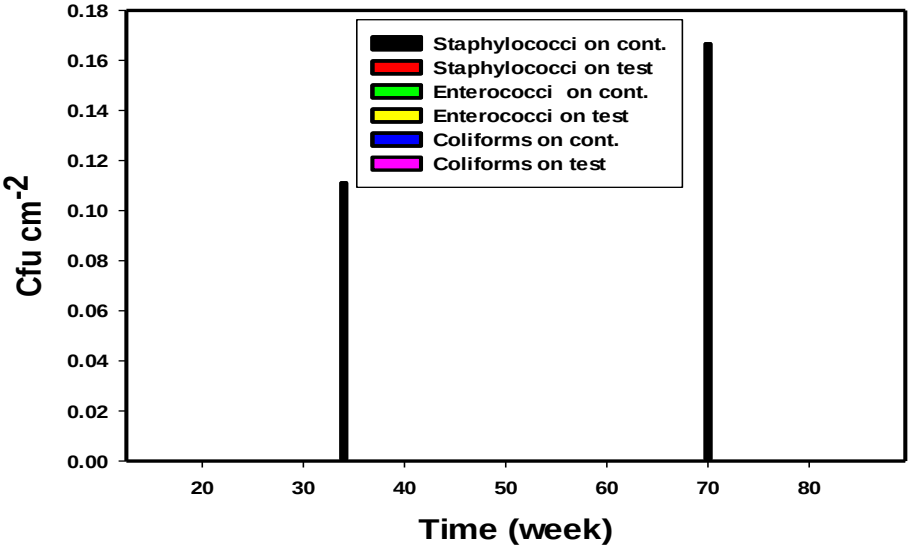

Figure 72b Total viable counts of indicator organisms (Staphylococci growing on Baird Parker Agar, Enterococci growing on Kanamycin Agar and Coliforms growing on Chromocult Coliform Agar)on coated Cu/SiO_2 tiles (test) and uncoated tiles (control) at site 2 at Manchester Royal Infirmary (ward 37)

Table 13 Summary of reduction in surface count of CVD samples (Cu/SiO_2 tiles) compared to control surfaces placed in Manchester Royal Infirmary ward 37 at site 2

Reduction in surface count of CVD samples (Cu/SiO_2) placed at site 2 ward 37 at Manchester Royal Infirmary (%)						
Samples	**week 20**	**week 28**	**week 36**	**week 44**	**Week 72**	**Overall mean**
Total cfu/cm	**75.4**	**0**	**100**	**0**	**100**	**68.3**
Staphylococci	**-**	**-**	**100**	**-**	**100**	**100**
Enterococci						**-**
Coliforms	**-**	**-**	**-**	**-**	**-**	**-**

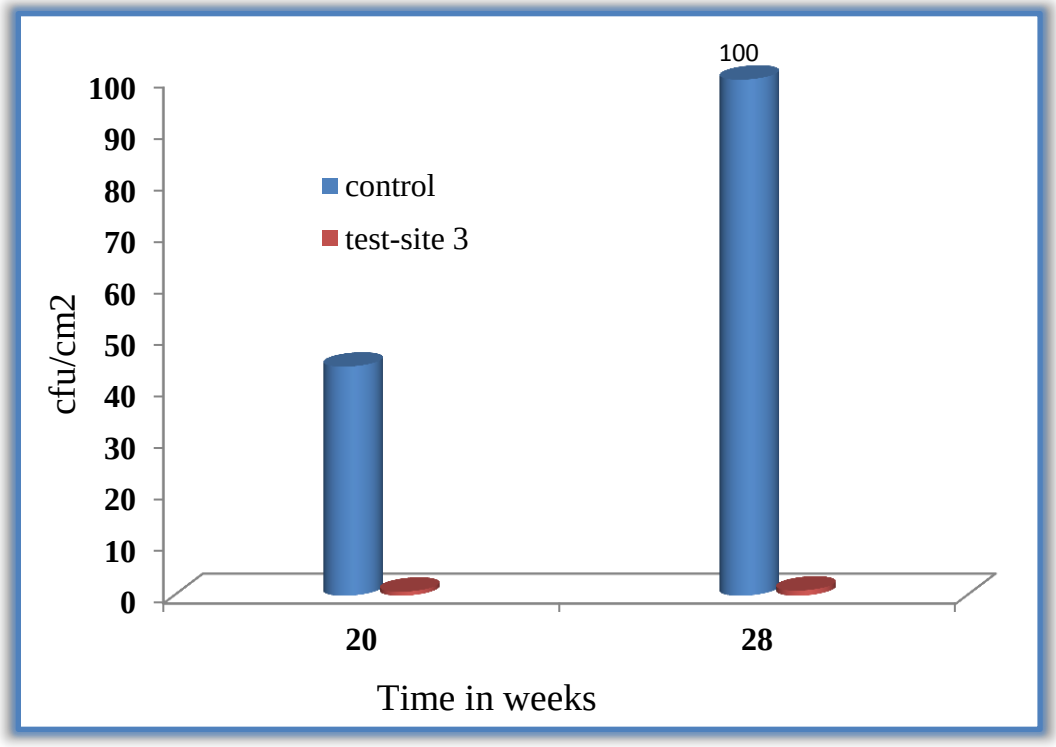

Figure 73a Total viable counts on coated Cu/SiO_2 tiles (test) and uncoated (control) tiles at sites 3 at Manchester Royal Infirmary (ward 12)

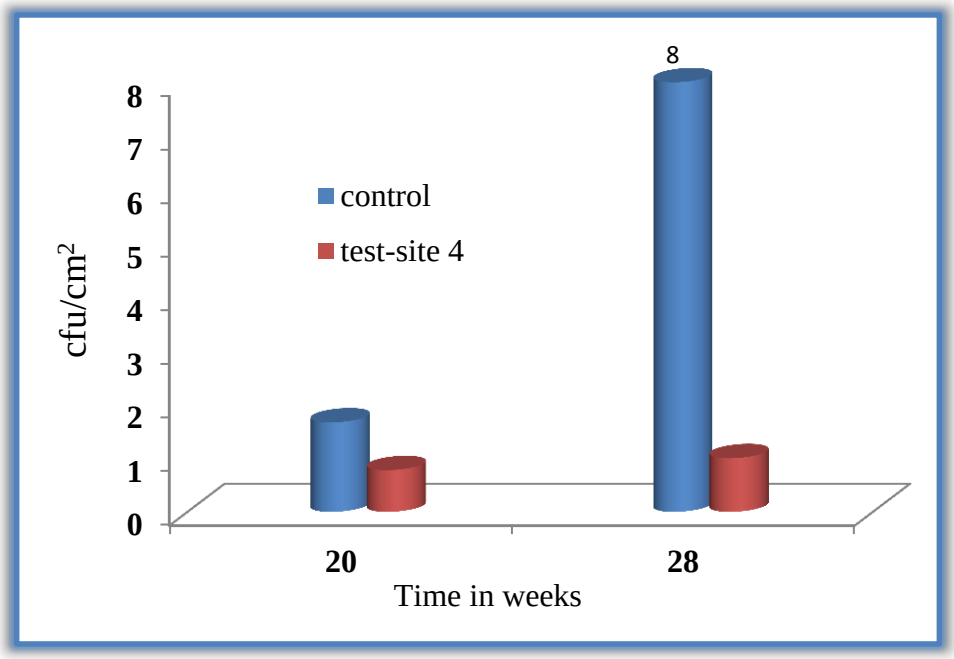

Figure 73b Total viable counts on coated Cu/SiO_2 tiles (test) and uncoated (control) tiles at sites 4 at Manchester Royal Infirmary (ward 12)

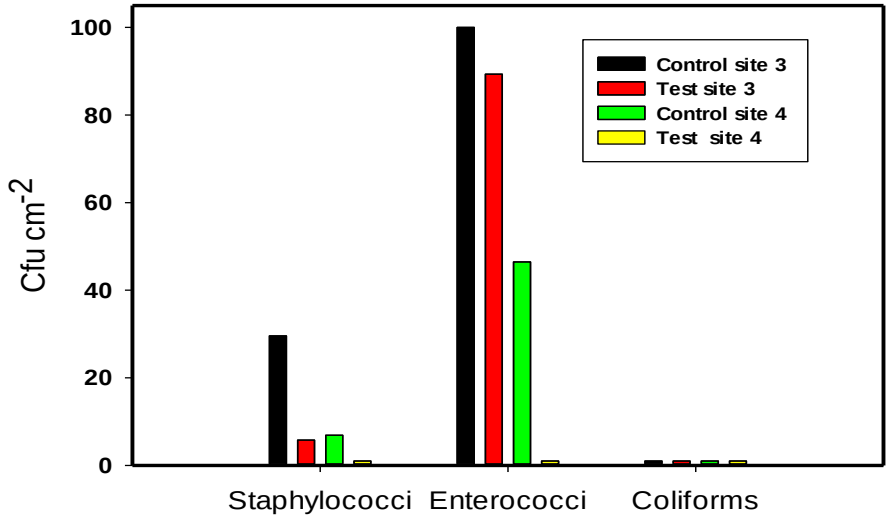

Figure 73c Total viable counts of indicator organisms (Staphylococci growing on Baird Parker Agar, Enterococci growing on Kanamycin Agar and Coliforms growing on Chromocult Coliform Agar)on coated Cu/SiO_2 tiles (test) and uncoated tiles (control) at sites 3 and 4 after 28 weeks at Manchester Royal Infirmary (ward 12)

Table 14 Summary of reduction in surface count of CVD samples (Cu/SiO_2-tiles) compared to control surfaces placed in Manchester Royal Infirmary ward 12 at site 3

Reduction in surface count on CVD samples (Cu/SiO_2) placed at site 3 at Manchester Royal Infirmary ward 12 (%)			
Samples	**week 20**	**week 28**	**Over all mean**
Total cfu/cm	**73.3**	**87.5**	**85**
Staphylococci	**-**	**80.4**	**-**
Enterococci	**-**	**10.6**	**-**
Coliforms	**-**	**0**	**-**

Table 15 Summary of reduction in surface count of CVD samples (Cu/SiO_2-tiles) compared to control surfaces placed in Manchester Royal Infirmary ward 12 at site 4

Reduction in surface count of CVD samples (Cu/SiO_2) placed at site 4 at Manchester Royal Infirmary ward 12 (%)			
Samples	**week 20**	**week 28**	**Over all mean**
Total cfu/cm	**98.25**	**99**	**98.7**
Staphylococci	**-**	**85.4**	**-**
Enterococci	**-**	**97.8**	**-**
Coliforms	**-**	**0**	**-**

5.3 Discussion

In an attempt to evaluate the *in situ* performance of the coatings, two experiments were conducted. Firstly this was done by placing different coated samples in a toilet facility. Secondly, copper coated tiles were placed in two different wards in Manchester Royal Infirmary. In the case of the toilet experiment, samples were cleaned 3 times over the period of testing, but in the hospital situation, samples were subject to the routine hospital cleaning protocol (1% chloroclean, 1-2 x per week).

5.3.1 In Manchester Royal Infirmary

The use of copper surfaces to control or reduce the microbial burden (MB) has recently been extended to reducing the nosocomial and environmental bio burden on touch surfaces in healthcare facilities (Marais *et al*., 2010). Compared to the extensive laboratory investigations into the efficacy of copper in the contact killing of microbes, *in vivo* studies are limited (O'Gorman and Humphreys, 2012). Although there was no standard level of surface contamination, it has been suggested that the number of viable cell count present on open, dry surfaces in hospitals can vary between 0 and 100 cfu/cm^2 (Griffith *et al*., 2000). In this study, there was a considerable reduction in the total colony count on copper surfaces on both wards, and copper surfaces were less likely to be contaminated with indicator organisms such as coliforms, staphylococci and enterococci. The present study differs from those previous studies in two respects. Firstly, sampling was performed over a longer period of time (16 months), and secondly the copper surfaces were not in direct contact with patients but still presented a risk to them through HCWs. Similar results were recently reported by Schmidt *et al*.,(2012a). However, their study was on medical devices in close proximity to the patients and was used routinely during direct patient care in an intensive care unit. Results of the present study also showed that the level of contamination was different over the period of testing, not only on control

surfaces, but also on copper surfaces; for example, the reduction of the total level of cfu in weeks 36 and 72 (ward 37) was almost 100% on both sites, whereas on week 28 there was no reduction on the site 2 (0%) and 67.6% on the site 1. This may be explained by two different possibilities: the type of bacteria that contaminate the surfaces, since the ability of bacteria to resist to copper is different from one strain to another as shown in this study and in many studies in the literature; and/or due to bacteria being deposited on the surfaces immediately prior to /or in a short period before sampling, as the activity of copper, like that of other antimicrobial substances, is not rapid (Casey *et al*., 2010). A concentration of less than 250 of aerobic colony count (ACC) per100 cm^2 (2.5/cm^2) of surface area, and any indicator organisms of 100 cfu/100 cm^2 (<1 cfu/cm^2) have been proposed as a standard for being considered benign, immediately after terminal cleaning (Dancer, 2004). If the ACC increases above this level, the transmission rate from the surfaces to healthcare workers and/or patients will increase (Schmidt *et al*., 2012a). In the present study, the levels of antimicrobial activity of copper coated tiles were equivalent throughout the study period. This was evident from the observation over the period of sampling, in that most of the copper tiles had no recoverable indicator organisms, or had a reduction in the total cfu of the indicator organisms compared to the control tiles.

The use of antimicrobial copper coated surfaces does not require additional training or supervision unlike other programmes designed to improve compliance with infection control, such as hand hygiene and patient screening. In fact it does not require alterations to existing cleaning practices or add to the annual environmental cleaning costs, as do other applications such as UV light or hydrogen peroxide vapour deposition for reduction in Microbial Burden (MB) (Mikolay *et al*., 2010, Salgado *et al*., 2013).

The results suggest that, even though there was reduced activity in both total count and indicator organisms over a long period of time, the coatings may have applications in reducing surface

bacterial contamination in real life use, where the surfaces are touched by hundreds of people such as in healthcare settings, schools and travel terminals. Indeed, using such surfaces in combination with regular cleaning and infection control practices may result in significantly lower MB and potentially safer surfaces.

5.3.2 In the ladies toilet

The presented data clearly indicate that the tiles performed well for the first four months, giving 95-99% reduction in surface contamination compared to control surfaces. This was also evident on other coated surfaces (glass and stain steel) coated surfaces. However activity was reduced after cleaning, which may have left residual detergent and soiling on the tiles, and may have spread contamination from the control tiles to the test tiles. The surfaces were then cleaned again and rinsed with sterile water to try to ensure that any contamination was completely removed. This restored activity, but the levels of contamination were much lower than in the first few months. Overall, the performance of the surfaces appeared to be better with higher levels of contamination. Stainless steel is usually used in kitchenware, medical equipment and food processing and storage facilities. However, microorganisms can exist on these surfaces. In a study conducted in a hospital in Hamburg, Germany over a period of 16 weeks, touch surfaces such as doorknobs, push plates and light switches in the patients' bedroom, rest room, and staff room were replaced with new surfaces composed of copper alloys (Mikolay *et al*., 2010). These surfaces were cleaned every morning using the surface disinfectant Incidin ®PLUS Results show that the total number of cfu on copper was reduced by 63% compared to the control (aluminium) surfaces. However, the cfu was increased after cleaning both surfaces (copper and aluminium). This may due to the residual of the detergent used to clean the surfaces. Incidin ®PLUS contains glucoprotamin as the active ingredient, with copper ions working synergistically with quaternary ammonium compounds to kill bacteria. So, rather than

increase the killing ability of copper, the glycol may have generated a thin layer between the copper surface and the bacteria, which might have led to an increase in the copper toleration of the bacteria (Mikolay *et al*., 2010). This may explain the reduction in the efficacy of the copper surfaces seen in the present study after cleaning, compared to their activity before cleaning, since the coated surfaces were cleaned using Tri Methylnonyl Polyethylene Glycol (Tergitol) which also contains glycol as an active substance. Moreover, some samples in this study maintained their activity after repeated cleaning with sterile fluids. For example, after 18 weeks, samples 5 and 2C (painted stainless steel) which had the highest concentration of copper compared to the other stainless steel samples, showed a 95% - 97% reduction compared to the control surfaces, but after the first cleaning, sample 2C lost its activity and sample 5's activity was reduced by 60%. However, both samples maintained their activity after the second cleaning, but with a lower efficacy compared to their activity after the first swab. However, sample 5 lost its activity again after the third wash. This loss of activity may due to the water damage that occurred to the board for some unknown reason as shown in Figure 62. In fact, this may also due to high amount of copper removed by wash cycles, since tiles with high amount of copper are more sensitive to wash than tiles with small amount of copper as we shown in washability test in chapter 4 (Figure 47). A comparison study between the re-cleaning ability of copper and stainless steel after several soiling cycles was done using two different cleaning agents recommended by UK National Health Service guidelines (1% sodium hydrochlorite is used to disinfect surfaces contaminated with body solutions such as proteins or blood, and 70% industrial methylated spirit is used to clean open hard surfaces) (Airey and Verran, 2007). Surfaces were inoculated with a known concentration of bacteria suspended in bovine sodium albumin as an example of residual matter. The results showed that both materials were easily cleaned after the first soiling cycle, but a build-up of cells was observed on copper surfaces after several cleaning cycles, whereas stainless steel remained highly cleanable. The accumulation of material on copper is

probably due to the high reactivity of copper, resulting in surface conditioning (Airey and Verran, 2007). On the other hand, it was reported that copper surfaces remained active when soiled with two different strains of *C. difficile* (vegetative cells). In this study, cells were suspended in protein to simulate a contaminated clinical environmental (Wheeldon *et al*., 2008). However, this study differs from that of Airey and Verran (2007) in that their investigation evaluated the antimicrobial effects of copper alone without including a cleaning agent. In fact, how soiling and cleaning affects the antimicrobial properties of copper surfaces has not yet been studied in detail (Grass *et al*., 2011). For this, it is important to select the appropriate cleaning or disinfecting protocols for selected surfaces, as standard hygiene procedures used in hospitals may help to reduce the contamination level of touch surface as showed by many studies.

These *in situ* results also showed that the activity of silver coated tiles and copper coated tiles were initially almost equal. Both tiles showed a reduction in terms of cfu of 98% for the first period of testing. Both lost their activity after a first wash, and then retained it after a second wash. However after a third wash, silver tiles lost all their antimicrobial activity which may due to two different reasons; silver come off from the surfaces (due to poor durability of silver coated as stated before, Figure 42c in chapter 4) and the water damage that occurred to the board. In contrast, *in vitro* copper was more active than silver. However, comparisons between the activities of surface *in situ* to their activity *in vitro* were not possible, since in vitro, a certain concentration of known bacteria was used. It has been reported that silver was not as active *in situ* as copper was. The efficacy of material containing silver was investigated at a temperature and humidity typical of an indoor environment as seen in hospitals (~24% RH and 20ºC) (Wood *et al*., 2007). The results showed that stethoscope diaphragm covers with a material containing silver ions had a mean colony count of 246.5 per sample compared to 71.4 per sample in the case of uncovered stethoscope diaphragms. It is predicated that the added silver cover may provide a surface that protects microbes from cleaning

agents, thus creating a higher colony count (Wood *et al*., 2007). This lack of efficacy in the case of silver may explain in part why silver containing materials have not received a permit from the US Environmental Protection Agency (US EPA) to be used in public health sectors as is the case with regard to copper. Copper and its alloys have been awarded US EPA registration as the first solid material to be used in a public health sectors after demonstrating its strong antimicrobial efficacy against pathogenic bacteria (Michels *et al*., 2009). This is another strong reason for chosen Cu/SiO_2 for hospital testing. The efficiency of copper alloys as an antimicrobial agent against a wide variety of pathogenic microorganisms has been reported. Literally, most metal ions own a strong or weak bactericidal ability. For example, Fe and Ni possess a bactericidal ability. However, most alloys containing these elements do not show bactericidal ability. The main reason is that there are no active particles on the alloy surfaces. Indeed Cu and Ag are most suitable as antibacterial agents (Zhang *et al* 2012a). In a comparison study to assess the ability of different copper alloys (25 samples) and stainless steel to inhibit the growth of *E. coli* O157: H7 and *L. monocytogenes* which are known as food- borne pathogen, Michels *et al*. (2005) found that all the copper alloys tested possessed antimicrobial activity against *E. coli* O157: H7 and *L. monocytogenes*, and this activity increases with the increased copper content of the alloy. In contrast, the stainless steel which is a common material for food equipment had no effect on bacterial growth (Michels *et al*., 2005). Moreover, it has been shown recently that *K. pneumoniae* can be killed within 60 min on pure copper (99.9%Cu) and copper alloys (brass containing 70% Cu and zinc 30%; copper nickel 90% Cu and Ni 10%) but the killing was delayed on nickel silver (after 270 minutes) which contained a lower concentration of copper (Cu 55%), while *P. aeruginosa* was killed after 180 min (on pure copper) and 270 min on copper alloys. On the other hand, 180 min was required to kill *A. baumannii* on all copper surfaces (Mehtar *et al*., 2008).

The presented data clearly indicate that the use of antimicrobial hard surfaces for touch surfaces, and copper in particular, decreases the number of living bacterial cells adhering to these surfaces. Therefore these surfaces may be useful in real life use. However, additional studies on the effect of the use of detergents on microbial activity on such surfaces are needed, since their efficiency is critical with regard to inducing surface activity. Any improvement to durability will need modifications to the coating conditions.

General discussion

6.1 General discussion and conclusions

The results in this study show that all three types of surface, TiO_2-Cu, SiO_2-Cu and SiO_2-Ag were highly active against the standard test strains of bacteria recommended in the BS ISO22196 and BS ISO 27447 tests but were also active against clinical isolates although with reduced activity in some cases. For example MDR pathogenic bacterial strains such as MRSA and ESBL-producing *E. coli* showed more resistance to copper surfaces (copper/silica and copper/ titanium) than their sensitive-type strains. However, this issue is not addressed by the BS method which uses very sensitive strains to allow comparisons to be made between different surfaces. Neely and Maley (2000) showed that there was no difference between the viability of vancomycin-sensitive and vancomycin-resistant *E. faecalis* and *E. faecium* on plastic (polyethylene) and fabric surfaces. Santo *et al*. (2010) showed that some resistant strains (*Pseudomonas oleovorans* and *Micrococcus luteus*) isolated from copper coins (which presumably survive on copper coins due to soiling) survive longer than their type strains (sensitive strains) on moisture pure copper surfaces but are not resistant to ionic copper. It is therefore unlikely that copper ions and copper surface resistances are controlled by the same mechanisms, since metallic copper resistant isolates from coins were sensitive to dissolved copper ions. In addition, there were no survival differences on dry copper, suggesting a different bactericidal mechanism. Whether or not the ability of resistant strains to resist copper death is related to their ability to resist antibiotics needs further study.

In this study, a modification of the BS ISO 22196 method was used to allow determination of death rates using room temperature to reflect activity at normal temperatures. The incubation temperature in BS, which is 35°C, is not the typical temperature present in health care settings. The results suggested that activity was increased at higher temperatures, giving an inflated impression of activity

compared to room temperature. This method does not reflect the natural contamination that may occur *in situ*, and there is an urgent need for standardised methods that reflect this as has been suggested by previous authors (Grass *et al*., 2011, O'Gorman and Humphreys, 2012). In fact, in the present study, bacterial growth on the control surfaces was also affected. This may be due to the effect of temperature on the control surfaces (SiO_2), as we notice that silica had some antimicrobial effect, which may increase with higher temperature. Another drawback of the BS method is that the level of contamination is very high (approx. 10^6 cfu/cm^2) and deposited onto a small surface area. This level of contamination is several times higher than the level of pathogens commonly present on many surfaces that require cleaning, thus giving unrealistic results. In fact, this is much higher than levels of contamination that have been measured in outbreaks (<10^2 cfu cm^2: Otter *et al*., 2011). The use of a bacterial suspension will allow diffusion of the antimicrobial (e.g. Cu^+ or Ag^+) from the surface; this would be reduced if the contamination were dry or dried quickly (by using a small amount of bacteria suspension). In addition, the majority of studies in the literature used standard test strains and/or specific types of microorganisms. Finally, this method is not an appropriate test for microbial spores, cysts, and biofilms, which have a much greater resistance to disinfection treatments due to their structure compounds (Dunlop *et al*., 2010). Most of the above problems were also associated with the BS ISO 27447 photocatalytic method. Controls in both BS methods remained viable for up to 24 h with only one or half log reduction. This may be due to the low concentration of nutrient broth (1/500) used in the re-suspension medium, which meant that the cells were less stressed and remained viable for longer. The oxidisable material present in the re-suspension medium will compete with the bacteria for ROS. Although the use of distilled water instead of NB for such tests does stress the bacterial cells, it will eliminate any variation in interference effects from ions and organic matter, which are likely to vary for different surfaces (Foster *et al*., 2010). The presence of microbes on surfaces may be associated with proteins. However, this fact is not

mentioned in BS methods. A protein inhibition study showed that as little as 1% bovine serum albumen (BSA) reduces the activity of copper surfaces, even those with high Cu content. Inhibition caused by the presence of protein in, e.g. serum or food, may be much higher. Increasing the amount of copper in the coating reduces the inhibitory effect of protein, but the hardness of the coating is also reduced. The makeup of the coatings used for *in situ* applications will need to hold a compromise between durability and activity (Varghese *et al*., 2013b).

All coated surfaces (Salford and OCAS) used in this study were prepared using the CVD method. However, different antimicrobial activity was seen: the activity of Salford samples was much higher than OCAS samples in both laboratory testing and *in situ* (toilet test). This may be due to the different techniques used to produce the coated surfaces. In Salford samples, copper/silica was deposited on borosilicate glass, and Tetraethylorthosilicate and copper sulphate were used as precursors for silica and copper respectively. In OCAS samples, multilayer films consisting of silica base layer, an intermediate copper oxide layer, and a silica top-layer were used. Hexa-methyldisiloxane (HMDSO) and copper nitrate Cu $(NO3)_2$ were used as precursors for silica and copper oxide. Copper kills microbes on contact, so the fast killing seen in Salford samples was due to close contact between the microbe and the copper, which lead copper ions to dissolve from the surfaces and interact directly with microbes. On the other hand, the delay or reduction in copper activity seen on OCAS samples may be due to the top silica layer, which may protect the microbes from direct contact with the copper surface, and may also reduce the amount of copper eluted from surfaces. The amount of copper that diffuses to the surface during coating has not been studied for these coatings but this will be important for food applications as it would be undesirable for copper to diffuse into food in contact with the surface. It has been shown that the structures in which Ag and CuO were layered on top of the TiO_2 led to higher biocidal activity than coated surfaces in which TiO_2 was layered on top. This may be because of the increased availability of Ag and CuO eluted

from the surfaces. However, the hardness and durability of coated surfaces with TiO_2 layered on the top were much greater than those of other surfaces (Foster *et al*., 2010).

Surface decontamination is a complex process, especially in hospitals, where multiple surfaces made of several different materials present a considerable challenge, and bacterial resistance greatly increases the extent of the problem. The growing resistance of microbes to disinfectants and antibiotics is a cause of concern throughout the world, especially in areas where these types of microbes are present due to the appropriate growth conditions such as in the food industries and in the health sector. The use of coated surfaces should greatly reduce the survival time of microbes. There is increasing interest in the reintroduction of copper and copper alloys into hospitals for their antimicrobial activity (Mikolay *et al*., 2010), but there is some evidence that antimicrobial activity may be reduced by conditioning of the surface, allowing a build-up of contamination (Casey *et al*., 2010). The residual self-cleaning activity of the CuO-TiO_2 coatings may help to prevent this build-up by oxidising any organic matter on the surface e.g. dead microorganisms, as previously shown by (Yates *et al*., 2008).

The durability of surfaces is one of the main surface features that should be considered in addition to their antimicrobial activities, since long life biocides are needed to ensure the overcoming of microbial resistance. Different studies have used different methods and different copper sources, which make their results hard to compare. Therefore, a standard production method of copper surfaces is urgently required to evaluate their antimicrobial efficiency. There is a strong need for better standard methods for testing the antimicrobial efficacy of antimicrobial hard surfaces to overcome the drawbacks of the current standard as described above. The comparison of results from different studies are very difficult, and may not even be possible due to different experimental

conditions (such as types of bacterial strains, growth media and growing conditions, including dry and wet inocula) as have been used in different studies (Foster *et al*., 2011).

Generally, the results of this study correspond with other data present in the literature (Wilks *et al*., 2005, and Santo *et al* 2008). Mainly in terms of the effect of copper on *E. coli*, from the results of the present study it is possible to conclude that copper has a strong effect on the growth of *E. coli* cells where a complete killing of >5 $\log_{10}$ was reached within 4 h whereas a >5 $\log_{10}$ killing of *E. coli* was reached within 45 min and 60 min in Wilks *et al*., 2005 and Santo *et al* 2008 studies respectively. However, the differences in results between this study and these previous studies may be due to the different methods used and/or the different bacterial load inoculated. A dry inoculation method was used in Santo (2008) and Wilks (2005) studies whereas a wet method was used in this study. Also a different *E. coli* strain was used; W3110 in Santo report, O157 in Wilks repot and ATCC8739 in this study. Another possible reason is the concentration of copper in the event that copper alloys were used. It has been widely noted that the killing kinetics of bacteria on copper alloys with a high concentration of copper, is faster than alloys with a low amount of copper. In addition, the exact amount of copper that is required to kill microbes is unknown, and subject to more investigation.

Since the high CFU used in this study are rarely found in real-life systems, it appears that CVD coatings (Cu/SiO_2 and Cu/TiO_2) could have an excellent biocide effect, and be effective in reducing bacterial growth for practical applications such as the formulation of various biocide materials. Although the activity of Cu/TiO_2 was stronger under UVA irradiation, their activity under the visible light range would greatly enhance their use in the health sector (infection control) and in the food industry, where contamination association with biofilm formation is common.

The results of *in situ* use indicate that copper containing surface coatings are a promising antimicrobial material since they show antimicrobial reduction under harsh conditions for a long period of time (however, Cu-SiO_2 coated surfaces were cleaned twice a week as was the case with hospital surfaces). These antimicrobial surfaces may help to prevent growth and the transmission of pathogenic bacteria on commonly touched surfaces used in the health care sector, food preparation industries and/or any touched surfaces in other crowded area such as transport stations or schools. However, it must be considered that further research in clinical trials showing a continued reduction in HCAIs rates needs to be reported, before the widespread application of copper contact surfaces could be recommended. In addition, extra cost, minimum percentage copper content, and effective cleaning protocols for copper surfaces should also considered (O'Gorman and Humphreys, 2012). The extra cost of coating ceramic tiles is in the region of 1€ m^{-2} not counting the capital costs of installing the coating equipment (Paul Sheel, personal communication).

Overall, the results of this study have proved the broad range of antimicrobial ability of copper under different environmental conditions in both *in situ* and in laboratory use. Therefore, the use of copper surfaces in association with effective hand hygiene and the routine cleaning of environmental surfaces, would greatly reduce HCAIs rates, especially those caused by bacteria, particularly as they can be applied to different materials e.g. glass, ceramic tiles and metals. They may also find applications in other situations where the control of microbial contamination is important e.g. in the food industry.

Although HCAIs will probably never be completely eliminated, the introduction of antimicrobial coated surfaces into the healthcare sector is an important factor in terms of maintaining a microbial-clean environment. Such antimicrobial surfaces should be permanent, hard-wearing and appropriate for hospital conditions. The surface coatings investigated in this study, if used with other

antimicrobial materials e.g. Cu touch surfaces and antimicrobial fabrics, should help to greatly reduce the microbial burden. These will, however, have to be used alongside other hygiene precautions e.g. hand-washing and careful aseptic techniques (Page *et al*., 2009).

6.2 Future outlook

Antimicrobial hard surfaces are likely to be used as an improved cleaning and disinfectant strategy, especially in health care settings where the growth of antibiotic resistant bacteria are a cause of major concern. The present study evaluated the antibacterial activity of three different antimicrobial surfaces against eight pathogenic bacteria that are known to cause hospital-acquired infection. The present work could be used to evaluate antimicrobial activity against other types of hospital-related pathogens such as *Candida albicans* which are also known to cause a large number of deaths, as well as enormous additional healthcare costs due to infection (Page *et al*., 2009). There is a lack of clinical trials assessing the role of copper contact surfaces in eliminating anaerobic spores, especially *C. difficile.* The decontamination of surfaces exposed to *C. difficile* spores by conventional cleaning methods is challenging. In addition, the beneficial effects of copper contact surfaces may have a significant effect.

Due to time and access restrictions, the work at Manchester Royal Infirmary was done in only two different wards, and only a small number of copper samples was used. In order to fully evaluate the *in situ* performance of the coatings a much more thorough study of their performance is needed in different hospital wards and at other sites where reduction in microbial counts is important e.g., food preparation areas and public toilet facilities, especially those used in transport e.g. airplanes, trains and boats.

The killing mechanism of copper on resistant strains is another point that should be studied further, because the reports in the literature tend to contradict each other. In addition, comparisons between copper-killing mechanisms with regard to resistant strains and their sensitive strains are also important to fully understand the killing function of copper.

Chapter 7

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Appendix

Publications

Some of the results from this study have already been presented as posters and as contributions to publications:

1. Howard A. Foster, Sajnu Varghese, **Souad ElFakhri**, Mohamed Abohtera, D. W. Sheel, P Sheel, F.J.(Eric) Bolton. Can *in vitro* tests reflect *in situ* activity of antimicrobial coatings? Poster presented at The IDRN 'HCAI of the future' workshop in Leeds. May23rd 2011.
2. Howard A. Foster, Sajnu Varghese, **Souad ElFakhri**, Mohamed Abohtera, D. W. Sheel, P Sheel, F.J.(Eric) Bolton Antimicrobial activity of Cu-SiO_2 coatings against hospital pathogens. Poster presented at The IDRN 'HCAI of the future' workshop in Leeds. May23rd 2011.
3. H.A. Foster, D.W. Sheel , P.Evans , P. Sheel, S. Varghese, **S O. Elfakhri**, M. Abohtera, F.J Bolton. Biocidal surfaces: Challenges for exploitation and the potential of dual-layer titania Ag- and CuO-titania films. Invited presentation at The Photocatalytic and Superhydrophilic Surfaces Workshop, PSS2011**,** Manchester Metropolitan University, 12th September 2011
4. Howard A. Foster, **Souad ElFakhri** ,Sajnu Varghese, Mohamed Abohtera, D. W. Sheel, P. Sheel, F.J.(Eric) Bolton. Novel antimicrobial coatings for reduction of transmission of disease via surfaces. Poster presented to SGM spring conference, Manchester 25-28 April 2013
5. Foster, H. A. Sheel D.W., Evans P., Sheel P., Varghese S., **Elfakhri S.O**., Hodgkinson, J.L., Yates, H.M. (2012) Antimicrobial activity of dual layer CuO-TiO_2 coatings prepared by CVD against hospital related pathogens. Chemical Vapour Deposition **18**, 140-146 DOI: 10.1002/cvde.201106978
6. Varghese S., **Elfakhri S.O.,** Sheel D.W., Sheel P., Bolton, F.J., Foster, H. A. (2013). Antimicrobial activity of novel nanostructured Cu-SiO_2 coatings prepared by chemical vapour deposition against hospital related pathogens. *J.Applied Microbiol.* 115:1107-1116. DOI: 10.1111/jam.12308
7. Varghese, S., **Elfakhri, S**., Sheel, D., Sheel, P., Bolton, F. & Foster, H. 2013a. Novel Antibacterial Silver-Silica Surface Coatings Prepared by Chemical Vapour Deposition for Infection Control. *Journal of Applied Microbiology,* 115**,** 1107-1116.

Chemical Vapor Deposition

DOI: 10.1002/cvde.201106978

Antimicrobial Activity Against Hospital-related Pathogens of Dual Layer CuO/TiO_2 Coatings Prepared by CVD**

By Howard A. Foster,* David W. Sheel, Philip Evans, Paul Sheel, Sajnu Varghese, Souad O. Elfakhri, John L. Hodgkinson, and Heather M. Yates

Photocatalytically active films of TiO_2/CuO grown by atmospheric pressure thermal (APT)CVD are investigated for activity against multiple antibiotic-resistant pathogens namely, Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, vancomycin-resistant enterococci (VRE), and a recent isolate of methicillin-resistant Staphylococcus aureus (MRSA), that have been shown to persist in the hospital environment. The bacteria are killed by UVA irradiation of the photocatalyst with a >5 log kill within 4–6 h except for the MRSA where a 2–3 log kill is obtained after 6 h increasing to >5 log after 24 h. There is antimicrobial activity in the dark which is enhanced by irradiation with fluorescent light. There is also activity at 5 8C under UVA, but activity is lower when fluorescent light is used for illumination.

Keywords: Acinetobacter, CuO, Extended spectrum b-lactamase (ESBL^b), E. coli, Klebsiella, MRSA, TiO_2, VRE

1. Introduction

There is increasing recognition that environmental contamination plays a key role in the transmission of infectious diseases in the healthcare setting.[1–4] Depending on the organism and environmental conditions, pathogens can survive on surfaces for prolonged periods of time.[5] Environmental contamination and environmental persistence has been shown to be important in the transmission of MRSA,[6,7] VRE,[8,9] Klebsiella pneumoniae,[10] Clostridium difficile,[11] Acinetobacter baumannii,[12] and norovirus.[13] Enhanced environmental cleaning has been shown to reduce rates of infection.[2,14]

The photocatalytic antimicrobial activity of TiO_2 has been known for over 25 years[15,16] and a wide range of microorganisms have been shown to be susceptible.[17] Despite their potential however, although TiO_2-coated materials have been widely used for their self-cleaning activities, there has been no widespread uptake for antimicrobial use in the healthcare sector.[18] There are a number of possible reasons for this, including the requirement for UVA irradiation for activation, potential poisoning of the surface by disinfectants, and shielding of the surface from activating radiation by organisms and contamination on the surface. Although modification of the TiO_2 catalyst by doping (with, e.g., metals or dyes) can give catalysts that are active in visible light,[19] their durability has not been extensively reported. We have previously shown that dual layer films of TiO_2/Ag and TiO_2/CuO grown by APT-CVD possessed strong antibacterial activity when tested against standard test bacteria with UVA irradiation,[20–22] however acceptance for clinical application requires demonstration of activity against clinically relevant organisms, not just standard test strains. Preliminary experiments showed that, although there was reduced activity against some hospital pathogens, e.g., MRSA, compared to standard test strains the coatings still gave a 95–99% kill.[23] Preliminary tests also showed that the CuO/TiO_2-coated surfaces had antimicrobial activity in the dark, which was enhanced in fluorescent light. We have extended the study by testing against a range of bacteria which produce highly undesirable environmental contamination. The activity in fluorescent light and dark activity of the coatings, and the effects of reduced temperatures on activity, are reported.

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2. Results and Discussion

2.1. Characterization of the films

2.1.1. Physical and Chemical Characteristics of the Catalysts

The visual appearance of two different batches of films is shown in Figure 1. Both sets of films had a transparent pale

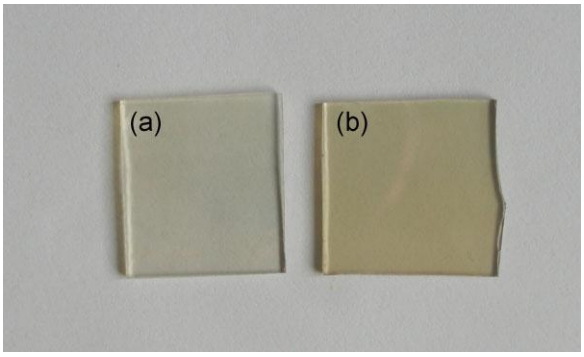

Fig. 1. Appearance of CuO/TiO_2 coatings a) sample 1, b) sample 2.

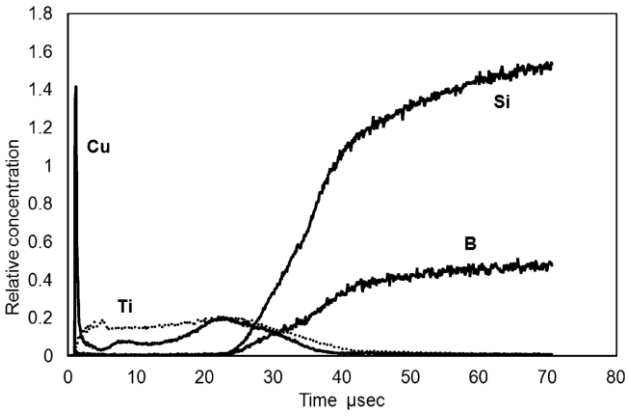

Fig. 3. GD-OES analysis of sample 2.

brown color with the second batch, prepared with a higher concentration of Cu, having a darker appearance. T values for visible light in the range 400–700 nm were 81.1% and 73.7% for samples 1 and 2, respectively (control glass was 91.5%).

Surface morphology was studied using scanning electron microscopy (SEM), see Figure 2. Both samples showed two types of crystalline aggregates, regularly spaced, large, bright aggregates of 240–390 nm (mean 338 _ 56 nm), smaller, darker crystals of 95–175 nm (mean 127 _ 28 nm) against a background of irregularly arranged rectangular crystals approx. 4 nm _ 4 nm _ 110 nm. The lower contrast for sample 2 is probably due to the lower acceleration voltage used (Fig. 2b). The visible characteristics are compatible with a mixed growth process of nucleation islands, gas-phase particulate, and standard second stage CVD film growth. Sample 1 was deposited at a lower temperature (550 vs. 580 8C) which may have influenced both nucleation and film growth. Energy dispersive X-ray (EDX) analysis showed that the larger aggregates had a higher concentration of Cu than the smaller darker crystals.

The glow-discharge optical emission spectroscopy (GD-OES) analysis showed a sharp peak of copper at the surface, decreasing rapidly then rising again to be relatively constant in the rest of the film (Fig. 3). Ti was only present in low concentrations at the surface but increased to a relatively constant level just below the surface. The results confirm the presence of Cu at the surface and suggest that the Cu may be concentrated in the larger aggregates shown by SEM. This is similar to our earlier observation,[21] and we propose that the labile components of the first layer diffuse through the growing film on top, the higher concentrations of copper species at or near the surface being thermodynamically favored.

An X-ray diffraction (XRD) pattern for sample 2 is shown in Figure 4. The trace shows peaks with 2u values characteristic of anatase (Joint Committee on Powder Diffraction Standards, 21-1272)[24] at 25.38 (101), 48.03 (200), 53.66 (105), and 54.78 (211), and weaker signals of CuO (Joint Committee on Powder Diffraction Standards,

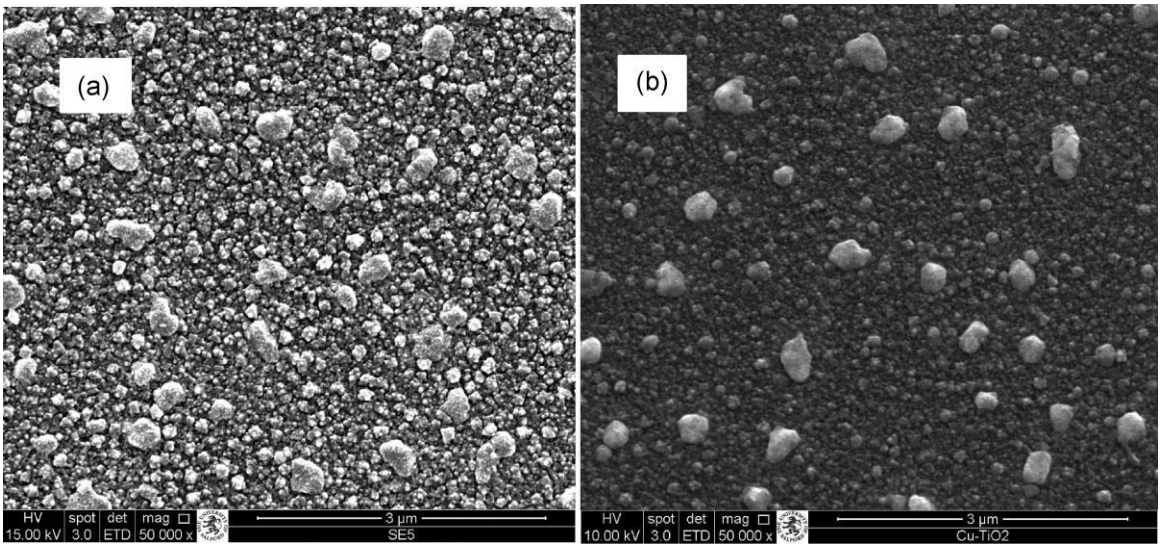

Fig. 2. SEM of CuO/TiO_2 dual layer catalysts. a) sample 1, b) sample 2.

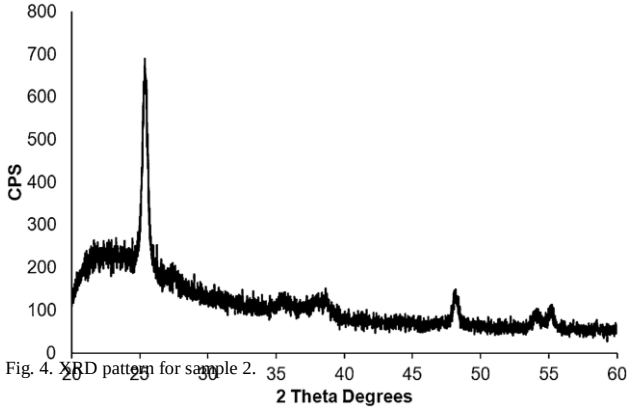

Fig. 4. XRD pattern for sample 2.

45-0937)[24] at 35.18 (11ı) and 38.1 (111). These show that the TiO_2 was crystalline and that a small amount of polycrystalline CuO was present.

From XRD it was not possible to confirm whether the sample consisted of two distinct layers or a mixture of materials on the surface. To determine the nature of the surface it was necessary to use X-ray photoelectron spectroscopy (XPS) as this is surface sensitive, only penetrating approximately the first 5 nm of the surface. A wide elemental scan established that the only major impurities were from carbon which was present on all samples and hence was used as reference at 285 eV for the binding energy shift.

The high-resolution surface scan for Cu 2p showed the position of the $2p_{3/2}$ at 934.6 eV with a splitting of D ¼ 19.9 eV, along with the shake-up satellites. Due to the positioning and the high intensity of the satellites (relative to the 2p) the species can be assigned to $Cu^{II}O$.[25] The equivalent scan for Ti 2p gave the $2p_{3/2}$ at 458.8 eV and a splitting of D ¼ 5.8 eV. This, along with the position of the main O 1s signal at 529.3 eV, confirmed that this was TiO_2.[26] The positions of the Ti 2p and Cu 2p were not shifted in comparison to standard single layers, suggesting that the species were chemically distinct.

In summary, the surface contains both CuO and TiO_2, suggesting that the relatively high TiO_2 deposition temperature has enhanced rapid diffusion of Cu ions though the growing layer to the surface. This was also confirmed in our earlier work using both XPS and Rutherford backscattering (RBS).[21]

Durability tests showed that both samples passed the tape test. This suggests that the coating adhered well to the glass substrate. As previously shown,[27] it is possible that the presence of copper oxide has enhanced adhesion. Thus the films may be durable in real use situations, however durability is also related to the hardness of the coating. Preliminary results suggest that the hardness of the film is reduced with increasing amounts of CuO and this is currently being investigated.

2.2. Antimicrobial Activity Against Hospital Pathogens

The activity of the catalysts against the standard test strain of E. coli (ATCC10536) using 0.24 mW cm^{-2} UVA irradiation is shown in Figure 5a. There was a 4 log reduction in viability after 4 h increasing to >5 log reduction after 6 h. This was rather slower killing than seen previously,[23] but may reflect the different structure of the second batch of films (see above). The catalysts were equally active against the Kpc^{þ} (K. pneumoniae carbapenemase producing) K. pneumoniae and again gave a >5 log reduction after 6 h (Fig. 5a). The activity against an ESBL^{þ} strain of E. coli is shown in Figure 5b. The killing curve was broadly similar to the standard test strain of E. coli with a >5 log kill after 6 h. A. baumannii is a Gram-negative bacterium that is able to survive in the environment for prolonged periods,[28,29] however it was more sensitive than E. coli, and a >5 log kill was obtained after 4 h (Fig. 5b). VRE have been shown to survive in hospitals for over a year.[28,30] VRE and MRSA are Gram-positive organisms which are mostly more resistant to photocatalytic killing than Gram-negative bacteria,[17] however the VRE was as sensitive as E. coli with a >5 log reduction after 6 h (Fig. 5c). The MRSA1595 strain was more resistant with only a 3 log reduction in viability after 6 h, however this still represents a 99.9% reduction, and a >5 log kill was obtained after 24 h (Fig. 5d). These results show that the coatings are active against a number of pathogens which have been shown to persist in the hospital environment following contamination by an infected patient, and presenting a risk of infecting subsequent patients in the same room(s). The advantage of antimicrobial surfaces is that activity is continuous whereas surfaces rapidly become recontaminated after conventional disinfection.

There is increasing interest in the reintroduction of copper and copper alloys into hospitals for their antimicrobial activity,[31,32] but there is some evidence that the antimicrobial activity may be reduced by conditioning of the surface allowing a build-up of contamination.[33] The residual self-cleaning activity of the CuO/TiO_2 coatings may help to prevent this, as we have previously shown.[21]

2.3. Effect of Temperature on Antimicrobial Activity

The effects of low temperatures on the antimicrobial activity of the films were investigated by resting the Petri dishes with the coated glass samples on a bed of ice to maintain a temperature of approx. 5 8C. The activity against the ESBL^{þ} E. coli was reduced after 4 h with only a 1–2 log kill but still gave a >5 log kill after 6 h (Fig. 6a). The reduction in activity was greater when fluorescent light was used, and only a 3 log reduction was seen after 6 h. A similar reduction in activity against A. baumannii was seen with UVA irradiation with a 3 log reduction after 4 h increasing to >5 log reduction after 6 h (Fig. 6b). The reduction in

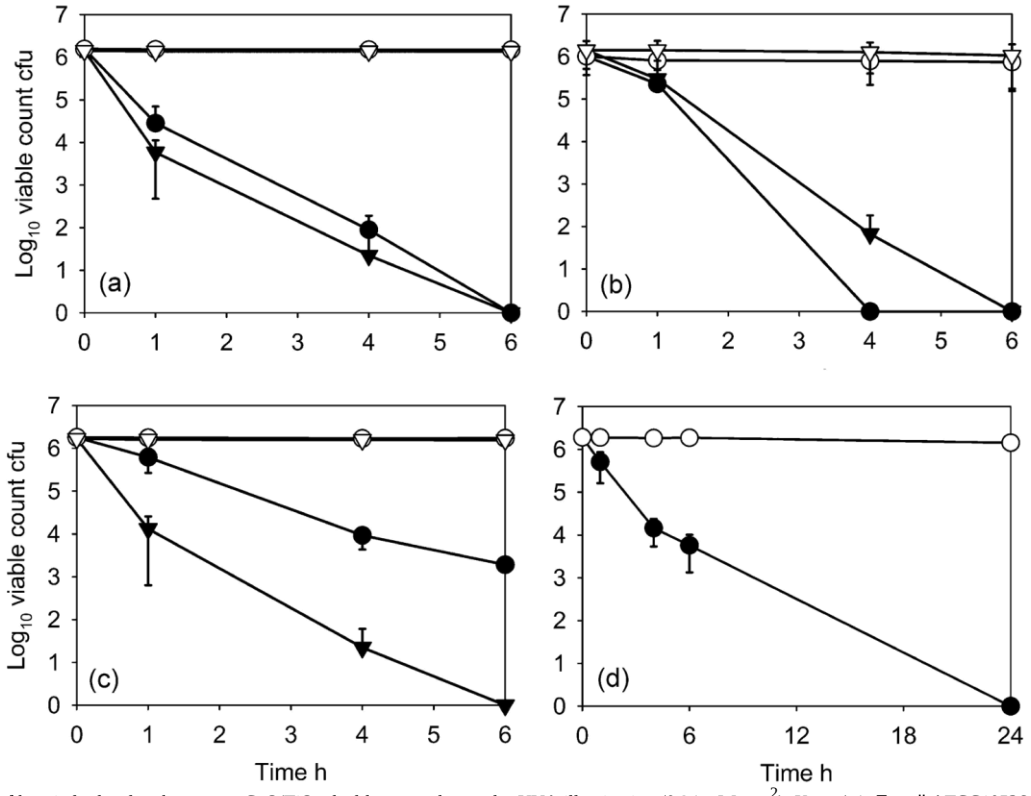

Fig. 5. Killing of hospital related pathogens on CuO/TiO_2 dual layer catalyst under UVA illumination (0.24 mW cm^{-2}). Key: a) *, E. coli ATCC10536; *, E. coli control; !, $Kpc^{þ}$ Klebsiella pneumoniae, 5 $Kpc^{þ}$ K. pneumoniae control. b) !, $ESBL^{þ}$ E. coli; 5, E. coli control; *, Acinetobacter baumannii; *, A.baumannii control. c) *, MRSA1595; *, MRSA1595 control; !, vancomycin resistant Enterococcus faecium; &, E. faecium control. d) Extended incubation for MRSA1595 *, MRSA1595; *, MRSA1595 control. Coating ¼ sample 2.

activity was again much greater with fluorescent illumina-tion, with a >5 log reduction after 6 h at 25 8C (Fig. 6a) but only 2 log at 5 8C (Fig. 6b). Cell killing probably occurs as a result of accumulation of damage to the wall/membrane until membrane integrity is impaired. The reduced activity may be due to slower production of reactive oxygen species (ROS) at 5 8C resulting in slower membrane damage. The results suggest that, even though there is reduced activity at lower temperatures, the coatings may have applications in reducing surface bacterial contamination in refrigerated areas, e.g., those used for food storage.

2.4. Effects of Illumination on Antimicrobial Activity

The effects of irradiation with UVA, fluorescent light, and dark activity on inactivation of E. coli ATCC10536 and the ESBL-producing strain are shown in Figure 7. The activity in

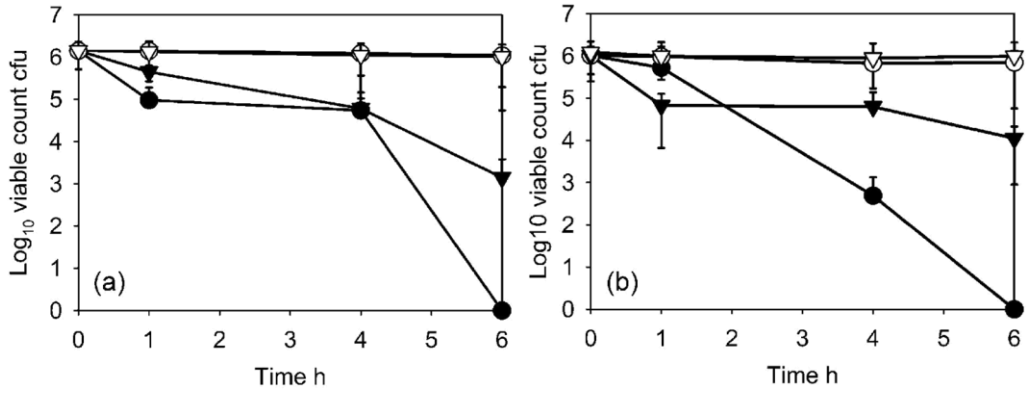

Fig. 6. Killing of $ESBL^{þ}$ E. coli and Acinetobacter baumannii on CuO/TiO_2 dual layer catalyst at 58C with different illumination. Key: a) E. coli, b) A. baumannii. *, UVA (0.24 mW cm^{-2}) *, UVA control; !, fluorescent light, 5 fluorescent control. Coating ¼ sample 2.

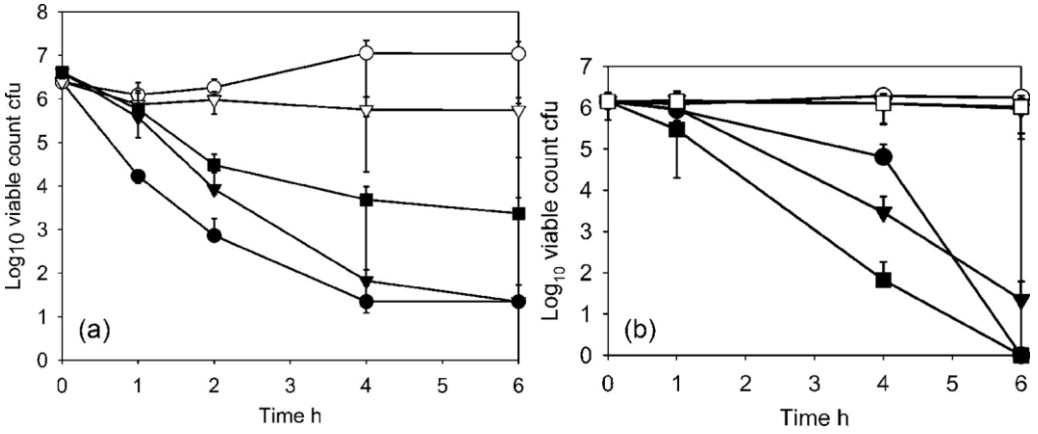

Fig. 7. Effects of illumination on killing of E. coli on dual layer catalysts a) Killing of E. coli ATCC10536 on Cu/TiO_2 catalyst sample 1. Key: *, Irradiated with fluorescent light (UVA approx 0.01 mW cm^{-2}); *, control; !, UVA 0.24 mW cm^{-2}; 5, UVA control; &dark. b) Killing of $ESBL^{þ}$ E. coli on catalyst sample 2 *, fluorescent light, * fluorescent control; !, UVA (0.24 mW cm^{-2}), 5, UVA control; & dark, & dark control.

the dark resulted in a 2–3 log reduction after 6 h for the standard strain on Sample 1 catalyst, increasing to 4–5 log kill with both fluorescent light and UVA at 0.1 mW cm^{-2} (Fig. 7a). The test for the ESBL-producing strain on sample 2 catalyst gave a >5 log kill after 6 h under all lighting conditions. A significant difference in activity was seen after 4 h ($P < 0.05$) with the order of inactivation UVA (0.24 mW cm^{-2}) > fluorescent light > dark activity. The count at 6 h for the fluorescent illumination of the $ESBL^{þ}$ strain represents just one colony on the three replicate plates, and is at the limit of detection for the method used. Low counts on the Petri dishes means that the results are subject to large errors.

The results show that it is possible to have photocatalytic antimicrobial activity upon exposure to fluorescent light, and that it is possible to utilize the low UVA at 365 nm and the higher emission peak of fluorescent lamps at 405 nm. This will enhance the usefulness of such coatings for applications in the healthcare sector.

2.5. Mechanism of Bacterial Inactivation in Fluorescent Light

Many previous studies have shown that the antimicrobial activity of photoactivated TiO_2 is due to the production of ROS.[17,34] TiO_2 is a semiconductor that can be activated by promotion of electrons from the valence band (vb) to the conduction band (cb). The energy for activation can be provided by irradiation of TiO_2 with light of sufficient energy to bridge the band gap. The promotion of electrons from the vb to the cb (e_{cb}^-) leaves a positively charged ''hole'' in the vb ($h_{vb}^{þ}$) (see Reaction 1 below). The electrons can migrate within the cb and the holes may be filled by an electron from an adjacent atom, the subsequent ''hole'' in that atom may again be filled from an adjacent atom and so on. Thus the holes can effectively diffuse through the material. Electrons and holes may recombine, an unproductive reaction or, when they reach the surface, they may undergo reactions to give ROS such as _OH (Reactions 2, 3) and $O_2\_^-$ (Reaction 4).

$$TiO_2 þ hy ð\_ \text{ band gap } ð3{:}0 \text{ eV} Þ ! e_{cb}^- þ h_{vb}^{þ} \quad (1)$$

$$h_{vb}^{þ} þ H_2O ! \_OH þ H_{aq}^{þ} \quad (2)$$

$$h_{vb}^{þ} þ OH_{ads}^- ! \_OHads \quad (3)$$

$$e_{cb}^- þ O_2 ! O_2^{—} \quad (4)$$

The superoxide can further react to give hydroxyl radicals and hydrogen peroxide (Reactions 5–8). Reaction of the ROS with microbial cells causes cell death as a result of oxidation of membrane lipids and disruption of membrane integrity, and may proceed to complete mineralization of cellular components (Reaction 9). Holes can also oxidize membrane components directly if cells are attached to the surface of the TiO_2.[35]

$$2O_2^{—} þ 2H^{þ} ! H_2O_2 \quad (5)$$

$$\_OH þ \_OH ! H_2O_2 \quad (6)$$

$$O_2^{—} þ H_2O_2 ! \_OH þ OH^- þ O_2 \quad (7)$$

$$O_2^{—} þ H^{þ} ! \_OOH \quad (8)$$

$$\_OH þ \text{cells} þ O_2 ! CO_2; H_2O \quad (9)$$

Activation of the TiO_2, however, requires photons with sufficient energy to bridge the band gap, approx 3.0–3.2 eV, thus light with a wavelength of <380 nm is required. There was a very low level of UVA in the fluorescent light used (<0.01 mW cm^{-2}) which may have been responsible for the

activity seen in fluorescent light, however CuO is a p-type semiconductor with a band gap of 1.7 eV,[36] which can be activated by light with a wavelength of <approx. 720 nm (Reaction 10). The photogenerated conductance band electron can then react with oxygen to give superoxide (Reaction 11) or reduce Cu^{II} to Cu^{I} in the matrix of the catalyst (Reaction 12) which can then generate superoxide radicals (Reaction 13) as suggested by Paschaolino et al.[37] Visible light may also promote interfacial charge transfer from vb electrons of TiO_2 to Cu at the junctions between CuO and TiO_2.[38]

CuO þ hyð< 660 nmÞ ! CuOðe$^{-}_{cb}$; $h^{þ}_{vb}$Þ

CuOðe$^{-}_{cb}$Þ þ O_2 ! CuO þ O^{-}_2

CuOðe$^{-}_{cb}$Þ ! CuOð$Cu^{þ}$Þ

CuOð$Cu^{þ}$Þ þ O_2 ! CuO þ $O_2^{—}$ (13)

Any Cu^{I} produced can react with oxygen to produce hydrogen peroxide which can then act as a source of hydroxyl radicals via a Fenton-type reaction.[39,40]

Production of ROS may also be enhanced by natural production, since they are produced as by-products of respiratory enzymes on the bacterial cytoplasmic mem-brane. If Cu^{I} is present intracellularly, this can result in production of _OH which can then damage intracellular molecules, especially DNA. Irradiation of water containing organic matter produces H_2O_2,[41] and _OH,[42,43] and superoxide is produced in the presence of copper ions.[44] Although these may not be produced in large amounts, they may all contribute to oxidative damage to bacterial cells.

3. Conclusions

Sequential deposition of thin films of CuO and TiO_2 by flame-assisted (FA)CVD and thermal CVD, respectively, led to the formation of a surface containing a mixture of both polycrystalline materials. The surface of the films contained both CuO and TiO_2, suggesting that the relatively high TiO_2 deposition temperature enhanced rapid diffusion of Cu ions though the growing layer to the surface.

These films were shown to be highly effective in killing resistant pathogens when illuminated with UVA. Acineto-bacter baumannii, $ESBL^{þ}$ E. coli, $Kpc^{þ}$ Klebsiella pneu-moniae and vancomycin-resistant Enterococcus faecium were reduced by >5 log after 4–6 h. Although MRSA was more fluorescent light. Photocatalytic activity of CuO was proposed as the mechanism of killing in fluorescent light.

4. Experimental

4.1. Production of the Coatings: Production of the coatings was by a two-step process. The Cu/CuO films were grown on glass 1 mm borosilicate glass (Corning Eagle 1737) using an AP-FACVD coater. In this process, flame energy is used to decompose the CVD precursors and when directed at a substrate (in this case scanned across) a film grows. Substrate heating was employed, which enhanced film density and adhesion. Sample 1 was produced as previously described [23]. A second batch (sample 2) was prepared with an increased amount of Cu as follows: The substrate temperature was set at 300 - C. An aqueous solution of 0.5 M $Cu(NO_3)_2$ was nebulized into a carrier of N_2 at a rate of 2 dm^3 $min^{–1}$ through the flame and onto the substrate with a propane/oxygen flame ratio of 1:20. The films were removed from the reactor and allowed to cool before subsequent reheating to 580 -C for TiO_2 deposition. The thermal CVD films were deposited using a custom-built APCVD reactor. The precursor for the TiO_2 deposition was titanium tetra-isopropoxide (TTIP; 107 -C, 1.0 dm^3 $min^{–1}$) transported to the reactor via a bubbler into 6.5 dm^3 $min^{–1}$ process gas.

resistant, numbers were still reduced by 3 log after 6 h increasing to >5 log after 24 h.

The films had killing activity in the dark which was enhanced by irradiation with fluorescent light. The films also had antimicrobial activity at 5 8C which was similar to that seen at 25 8C with UVA illumination, but lower with

4.2. Microorganisms and Growth conditions: E. coli ATCC 10536 and Staphylococcus aureus ATCC6538 were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen UK. $ESBL^{R}$ Acinetobacter baumannii, KPC^{R} (carbapenemase) Klebsiella pneumoniae, $ESBL^{R}$ E. coli, MRSA 1599, and VRE were obtained from the Health Protection Agency, Manchester, UK. Bacteria were sub-cultured onto Nutrient Agar (Oxoid, Basingstoke, UK) and incubated at 37 -C for 24 h. Cultures were re-suspended in Nutrient Broth (Oxoid) and kept on Microban[1] beads (TCS Ltd Merseyside, UK) at _80 -C. Prior to use, one bead was sub-cultured onto Tryptone Soya Agar (Oxoid) and incubated at 37 W 1 -C for 24 h. Bacteria were transferred to a second agar plate and incubated at 37 W 1 -C for 16 to 20 h. A loopful of test bacteria from this was dispersed in a 1:500 dilution of nutrient broth. The optical density of the suspension was adjusted to 0.01–0.02 at 600 nm in a spectrophotometer (Camspec, M330, Cambridge, UK) to give approx.

6.7 T 10^5 to 2.6 T 10^6 cells $mL^{–1}$.

4.3. Determination of Photocatalytic Antimicrobial Activity: Antibac-

terial activity was determined according to the method described in BS ISO 27447:2009 [45]. Coated and control glass samples were disinfected by shaking in 100% methanol for 20 min. The samples were removed aseptically and placed in a sterile container to allow evaporation of the methanol. Fifty microliters of cell suspension was inoculated onto the 20 mm T 20 mm sample and covered with a square of 1 mm borosilicate glass to ensure close contact between the culture and the film. The samples were placed in 50 mm diameter Petri dishes containing moistened filter paper to prevent drying out of the

suspensions. The samples were irradiated with Blacklight Blue lamps with a maximum UV light intensity of 0.24 mW cm^{-2}. Plain borosilicate glass was used for controls. Philips daylight fluorescent lamps were used for some experiments. Temperature of samples was approx. 25 -C during illumination. Samples were removed after 0, 1, 2, 4, 6, and 24 h and immersed in 20 cm^3 of Tryptone Soy broth and vortexed for 60 s to re-suspend the bacteria. A viability count was performed by serial dilution in saline, plating on nutrient agar in triplicate, and incubation at 37 -C for 48 h. Each experiment was performed at least three times and means and standard deviation determined. Uncoated plain glass samples were used as controls.

For some experiments the samples were kept on ice to maintain the temperature at approx. 5 -C during irradiation.

4.4. Characterization of the Films: For Scotch tape testing the film was cross-hatched every 5 mm with a diamond scribe. The adhesive tape was applied and pressed firmly to ensure consistent contact with the whole film. On removal the tape was observed visually and then under a microscope to determine if the integrity of the film had been maintained.

XRD measurements were performed using a Siemens D5000 with a Cu Ka source to obtain structural characterization of the films. The scans were run over the 2u range 15–60- at 0.02 increments using 5 s steps.

Surface composition of the films produced was analyzed by XPS. The system used consists of an Alpha 110 hemispherical analyzer and a monochromatic Al Ka radiation (hv ¼ 1486.27 eV) X-ray source (Vacuum Instruments). It was necessary to use a charge neutralizer as all the samples were insulating, due mainly to deposition on glass. This tends to shift the peak position, so measurements were referenced to the residual C 1s signal at 285 eV.

The relative copper content of the films with respect to depth was performed using a Jobin Yvon 5000 RF GD-OES using the following parameters; pressure 500 Pa, power 30 W, module 8.2 V, phase 2.75 V.

Surface morphology was investigated using SEM (Philips XL30) with samples sputter coated with a 2–3 nm layer of Pt/Pd to provide a conductive surface. The size of larger surface aggregates was determined by measuring 20 random aggregates and measuring at right angles. The smaller rectangular crystals were similarly measured for length, width, and height. Mean and standard deviations were reported. Elemental composition was determined by analysis of secondary electrons by a coupled EDX analyzer.

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ORIGINAL ARTICLE

Novel antibacterial silver-silica surface coatings prepared by chemical vapour deposition for infection control

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Abstract

Aims: Environmental contamination plays an important role in the transmission of infections, especially healthcare-associated infections. Disinfection transiently reduces contamination, but surfaces can rapidly become re-contaminated. Antimicrobial surfaces may partially overcome that limitation. The antimicrobial activity of novel surface coatings containing silver and silica prepared using a flame-assisted chemical vapour deposition method on both glass and ceramic tiles was investigated.

Methods and Results: Antimicrobial activity against a variety of bacteria including recent clinical isolates was investigated based on the BS ISO 22196:2007 Plastics – Measurement of antibacterial activity on plastics surfaces, British Standards Institute, London, method. Activity on natural contamination in an in use test in a toilet facility was also determined. Activity on standard test strains gave a $\log_{10}$ reduction of five after 1–4 h. The hospital isolates were more resistant, but MRSA was reduced by a $\log_{10}$ reduction factor of >5 after 24 h. Activity was maintained after simulated ageing and washing cycles. Contamination in situ was reduced by >99 9% after 4 months. Activity was inhibited by protein, but, although this could be overcome by increasing the amount of silver in the films, this reduced the hardness of the coating.

Conclusions: The coatings had a good activity against standard test strains. Clinical isolates were killed more slowly but were still sensitive. The optimum composition for use therefore needs to be a balance between activity and durability.

Significance and Impact of the Study: The coatings may have applications in health care by maintaining a background antimicrobial activity between standard cleaning and disinfection regimes. They may also have applications in other areas where reduction in microbial contamination is important, for example, in the food industry.

Introduction

Environmental contamination plays a major role in transmission of disease. Healthcare-associated infections (HCAI) are a major source of morbidity and mortality in both the UK and the USA (Klevens et al. 2007). Environmental contamination has been recognized as playing a role in transmission of HCAI (Hota 2004) and also food-borne infections (Todd et al. 2009). The importance of the environment in transmission of HCAI has been docu-mented for Acinetobacter baumannii (Aygun et al. 2002; Wagenvoort and Joosten 2002), Clostridium difficile (Kaatz et al. 1988), methicillin-resistant Staphylococcus aureus (MRSA; Dancer 2008; Rampling et al. 2001),

norovirus (Morter et al. 2011) and Vancomycin-resistant enterococci (VRE; Martinez et al. 2003). Due to the impact of hospital acquired infections, the number of publications proving the hospital environment as a major reservoir for transmission of such infections has increased (Aygun et al. 2002; Bartley and Olmsted 2008; Dancer 2009; Weber et al. 2010). Pathogens can survive on surfaces for days or even months depending on the organ-ism and environmental conditions (Kramer et al. 2006; Todd et al. 2009). VRE have been shown to survive for months on plastic surfaces and fabrics (Neely and Maley 2000), and one study showed environmental survival for nearly 3 years (Wagenvoort et al. 2011). In hospitals, patients are more likely to acquire HCAI if the previous occupant of the room had such an infection (Drees et al. 2008; Huang et al. 2006; Boyce 2007; Hayden et al. 2008; Nseir et al. 2011; Shaughnessy et al. 2011). Although early studies showed that routine cleaning was as effective as routine disinfection (Dharan et al. 1999) and that rou-tine disinfection had no effect on infection rates (Dasch-ner 1986; Danforth et al. 1987), recent studies show that enhanced cleaning and disinfection of the hospital environment does indeed reduce rates of infection (Dancer 2009; Kochar et al. 2009; Carling et al. 2010; Kleypas et al. 2011). However, surfaces can rapidly become recontaminated even after 'deep cleaning' (Hardy et al. 2007). The use of self-disinfecting surfaces may provide a solution to the recontamination problem. The antimicrobial properties of silver (Ag) are well documented (Clement and Jarrett 1994; Rai et al. 2009), and Ag is generally regarded as nontoxic (Williams et al. 1989). There are a number of products that are available that include silver as an antimicrobial agent. These include wound dressings and medical devices such as catheters (Edwards-Jones 2009; Page et al. 2009; Knetsch and Koole 2011), but there are relatively few reports of antimicrobial effects of silver-coated environmental surfaces, possibly because of cost and the soft, easily scratched nature of metallic Ag. This can partly be overcome by incorporating Ag into bulk materials or production of surface coatings. Replace-ment of frequently touched surfaces in a hospital ward with plastic impregnated with Ag gave an approx. 90% reduction in overall bacterial contamination (Taylor et al. 2009). There are a number of ways in which surface coatings of Ag and silica (SiO_2) can be prepared including sol-gel (Kawashita et al. 2000), powder coating followed by heating to fuse the silica (Esteban-Tejeda et al. 2012) and chemical vapour deposition (CVD; Cook et al. 2011). CVD has been widely used for many years across a wide range of industrial applications to produce thin film coatings. In such a process, a reactive gas mixture is introduced in the coating region, and a source of energy (usually thermal or plasma) applied to initiate (or accelerate) a chemical reaction, resulting in the growth of a coating on the target substrate (Choy 2003). The vari-ant of atmospheric pressure CVD (APCVD) has estab-lished itself increasingly in recent years, as a technologically and commercially attractive method for CVD coating. It has been particularly successfully employed in high-throughput continuous or semi-contin-uous coating processes in a wide range of industrial applications such as on-line glass coating, tool coating, ion barrier layer deposition, anticorrosion and adhesion layers on metals and antiscratch coatings on bottles. On-line CVD films are known for their hardness, which is a major advantage in subsequent industrial processing and in many of the target applications.

We have previously shown that thin antimicrobial coatings of SiO_2 and Ag prepared by CVD were hard and durable (Cook et al. 2011). Here, we report an extended investigation of the antimicrobial activities of such coatings.

Materials and methods

Micro-organisms and growth conditions

Staphylococcus aureus ATCC 6538, Escherichia coli ATCC 8739, Enterococcus faecalis NCIMB 775 and Pseudomonas aeruginosa NCIMB 10421 were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen UK. Pseudomonas aeruginosa AOH1 was obtained from water downstream of a wastewater treatment works and was from our own collection. Acinetobacter baumannii (extended spectrum b-lactamase, ESBL-producing), Klebsiella pneumoniae (Klebsiella pneu-moniae carbapenemase, kpc^+), methicillin-resistant Staph. aureus (MRSA) NCTC 12493 and Stenotrophomon-as maltophilia were obtained from the Health Protection Agency, Manchester, UK. Cultures were subcultured onto Nutrient Agar (NA, Oxoid, Basingstoke, Hants, UK) and incubated at 37°C for 24 h. Cultures were resuspended in Nutrient Broth (NB, Oxoid) and stored on Microban beads (TCS Ltd Merseyside, UK) at −70°C. Prior to use, one bead was subcultured onto NA and incubated at 37°C for 24 h.

Production of coatings

Silver-silica (Ag-SiO_2) coatings were deposited on 1-mm borosilicate glass (Dow Corning) as previously described (Cook et al. 2011) and 15-cm^2 white glazed ceramic tiles (Kai Group, Asparuh, Bulgaria obtained from B and Q Ltd, Chester, UK), using flame-assisted chemical vapour deposition (FACVD). The FACVD system was of in-house design and construction and consisted of a brass burner head above a translational stage and a precursor delivery system of ultrasonic nebulizer, bubbler and mass flow controllers (Cook et al. 2011). Tetraethylorthosilicate

was carried to the burner head using a nitrogen flow rate of 0 5 l min ^{1} from a heated and stirred bubbler (95°C 3°C, stirred at 120 rpm). An aqueous solution of silver nitrate (0 05 mol l ^{1}) was used as the silver pre-cursor and simultaneously delivered to the burner head by ultrasonically nebulizing the aqueous solution prior to carriage by nitrogen at 0 6 l min ^{1}. The number of passes under the burner head was six equating to a resi-dence time in the flame of approx. 12 s and gave a film approx. 25 nm thick.

In the later stages of the study, a new coating head was used, which was capable of coating 10-cm-wide sub-strates. Silver content of the films was varied by changing the concentration of the precursor and the flow rate to the coating head. The different conditions for the coat-ings are shown in Table 1.

Characterization of coatings

To assess the hardness of the deposited coatings, films were scratch-tested using a constant load scratch hard-ness tester. A diamond tipped scribe was moved through 50 mm over the surface with a 100 g load. The mean width of the resulting scratch over six points was then measured under 2009 optical magnification and com-pared with similar data from materials of known Mohs hardness (aluminium, steel, copper, glass and quartz), and Mohs hardness values of the deposited films were calculated. Results are the means of three determina-tions.

Adhesion of the coating to the substrate was deter-mined by Scotch tape testing. The coating was cross-hatched every 5 mm with a diamond scribe, and the adhesive tape was then applied and pressed firmly to ensure consistent contact with the coating. On removal, the tape was observed visually and then under a micro-scope to determine whether the integrity of the film had been maintained.

Table 1 Details of coatings used in the study

Coating number	Substrate	$AgNO_3$ precursor concentration mol l ^{1}	Flow rate to burner head l min ^{1}	Mohs hardness	Transmission%
1	Glass	0 1	2	ND	85 3*
2	Glass	0 05	2	5 9	88 5
3	Glass	0 1	2	2 3	85 3
4	Glass	0 5	2	1 6	66 5
5†	Glass	0 25	0 6	2 3	84 5
6†	Tile	0 25	0 6	NT	N/A

*Transmission of control glass 91 5%.

†New coater head. NT not tested, N/A not applicable.

Effects of washing and ageing

The effects of ageing on activity were determined by exposing the coatings to an accelerated ageing process in an in-house environmental chamber. This involved exposing samples to 4 h cycles of 1 h on with 100% humidity, 80°C and UVA at 2 mW cm ^{2} and 3 h off for a total of 25 cycles. The irradiation was from a 230 V 50/ 60 hertz Vilber-Loumat-T-4LN 4W bulb.

The effects of washing on activity were determined by washing the coated glass samples with a standard soap solution (International Products Corporation Micro-90 Concentrated Cleaning Solution diluted 10 fold with tap water to give a 2% solution), wiping with a soft cloth (MC-CLMR multipurpose microfibre cloth, TJM Clean-ing Services Ltd, Glossop, UK) and rinsing with warm tap water approx. 40°C. Slides were degreased with propan-2-ol and dried. This was repeated 1009.

Determination of film density

Transmission of the coatings in visible light was mea-sured using an Aquilla NKD7000 spectrometer using plane polarized light source and transmission averaged over the 400–700 nm wavelength range and measured at a 30° angle. The result was the mean of three measure-ments of the average value over the wavelength range.

Scanning electron microscopy

Surface morphology was investigated using scanning electron microscopy (SEM; Philips XL30, Eindhoven, Holland) with samples sputter-coated with a 2- to 3-nm layer of Pt/Pd to provide a conductive surface. Elemental composition was determined by analysis of secondary electrons by a coupled X-ray dispersive (XRD) analyser.

Testing for antimicrobial activity

Antimicrobial activity was tested based on BS ISO 22196:2007 (Anon. 2007) except that glass covers were used rather than plastic and samples were tested after dif-ferent times rather than 24 h as specified in the test. The test was also performed at room temperature 20–25°C rather than 35°C as preliminary tests showed that incuba-tion at 35°C gave inflated values of activity, and we expe-rienced difficulty in maintaining viability of the controls for more than 6 h with Staph. aureus. We felt that the activity at room temperature would more accurately reflect the in use activity. Twenty-millimetre square sam-ples of coated and control glass and 18 mm square covers were cleaned and disinfected by suspension in 90% meth-anol

for 20 min on an orbital shaker at 100 rpm. The squares were transferred to a sterile Petri dish and left for at least 1 h to allow the methanol to evaporate. Colonies were resuspended in a 1 : 500 dilution of NB and adjusted to OD 0 01–0 02 at 600 nm in a spectrophotom-eter (Camspec, M330, Cambridge, UK) to give approx. 2 9 10^7 colony-forming units (CFU) cm^{-3}. Fifty microli-tres was inoculated on to each test sample and covered with an 18-mm square of 1-mm borosilicate glass to ensure close contact between the culture and the film. The samples were placed in 50-mm-diameter Petri dishes containing moistened filter paper to prevent drying out of the suspensions. Plain borosilicate glass was used for controls. Samples were removed after 0, 1, 2, 4, 6 and 24 h, and both test slides and coverslip immersed in 20 cm^3 of sterile Tryptone Soy broth (TSB, Oxoid) and vortexed for 60 s to resuspend the bacteria. A viability count was performed by dilution and plating on NA in triplicate and incubation at 37°C for up to 48 h. As zero cannot be plotted on a logarithmic scale, one was added to each count to allow plotting zero counts. The samples were examined microscopically after resuspension to ensure that all bacteria had been removed from the surface.

For some experiments, bovine serum albumen (BSA: Sigma-Aldrich, Poole, Dorset, UK) was added to the medium used for resuspension of the test culture prior to inoculating onto test and control surfaces at final concentrations of 10, 50 and 100 g l^{-1}.

In situ testing

To investigate the performance of the coated tiles in 'in use' situations, two coated and two control tiles were mounted on board and exposed to natural contamination in the ladies toilet facility in the Peel Building of the University of Salford (see Fig. 5). Substrates were cleaned with methanol prior to starting the experiment to remove any contamination that had occurred while handling. The board with the samples was turned 180° three times per week on alternate days. The tiles were left in place for 2 weeks and sampled by swabbing 8 5 9 9 cm of the coated area of the sample. Swabbing was repeated after 2 months then after 3 and 4 months. Results are the means of two tiles.

Surface swabbing

Swabs (NRS™ Transwab containing 5 ml Neutralising Buffer, Medical Wire, Corsham, Wilts, UK) were moistened with the buffer and applied 159 horizontally and 159 vertically in zig-zag pattern over the surface, rotating the swab so that the entire area was sampled. We had previously determined that the neutralizing broth inactivated any Ag eluting from the surface. Swabs were immediately transferred to the neutralizing buffer tube and closed and taken to the laboratory. The swabs were agitated for 60 s on a vortex mixer and serial dilutions prepared using saline. One hundred microlitres from each dilution was inoculated onto TSA in triplicate. The plates were incubated at 37°C and colonies counted after 24 and 48 h. Colonies were counted and the count was adjusted to CFU cm^{-2}.

Results

Characteristics of the films

The films had a pale brown tinge (Fig. 1) that was darker in the films with a higher Ag content, for example, coating 4 (Fig. 1d). Transmission in the visible range was 84 5–88 5% compared with 91 5% for the control glass

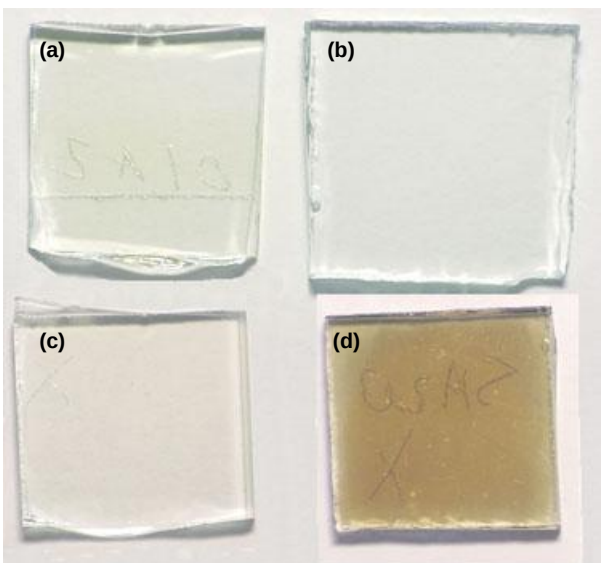

Figure 1 Visual appearance of coatings. (a) Coating 1, (b) Control glass, (c) Coating 3 and (d) Coating 4.

although this reduced to 66 5% in coating 4 with increased Ag content (Table 1).

Scanning electron microscopy showed an amorphous background with aggregates of Ag/AgO embedded in the surface (Fig. 2a). The films show silver nano-particulates embedded in the surface, which increased in number with increased silver concentration in the delivered gas phase. The size of the silver nanoparticles also increased with increased Ag content (coating 5 Fig. 2b). The results suggest that the silver aggregates grow by forming islands in the silica. With higher concentrations of silver, the islands coalesce and may reduce the ability of silica to bind the coating onto the substrate.

Hardness

The coatings with lower silver concentrations all passed the tape test, showing good adhesion to the substrate for low concentrations of Ag. However, the hardness decreased with increasing Ag content (Table 1). The photograph of coating 4 with the highest Ag content shows that it was easily damaged (small clear patches can be seen in Fig. 1d) and some of the coating was lost on the tape test.

Antimicrobial activity

The antimicrobial activity of the coatings (coating 2) is shown in Fig. 3. Escherichia coli 8739 was killed (log_{10} reduction factor of >5) within 1 h, and Staph. aureus 6538 (a methicillin sensitive strain, MSSA) was reduced by a log_{10} reduction factor of four after 1 h and completely killed after 4 h (Fig. 3a). The killing of Ps. aeruginosa was dependent on the strain (Fig. 3b). The disinfectant test strain NCIMB 10421 was as sensitive as the E. coli and was killed within 1 h, but the wild isolate AOH1 was much more resistant with only a log_{10} reduction factor of 3 after 24 h. The controls showed no

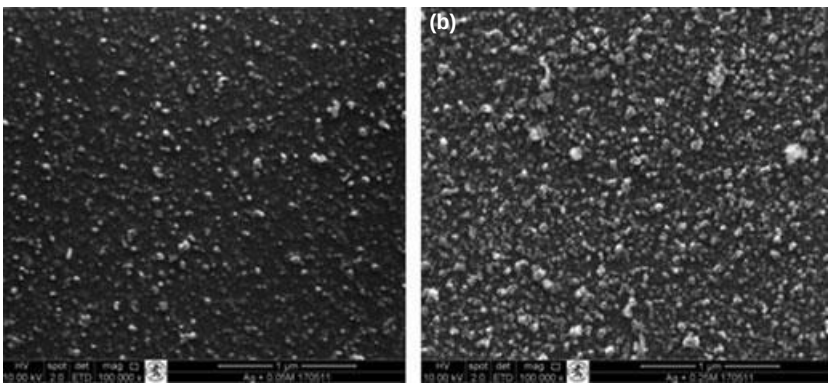

Figure 2 Scanning electron microscopy (SEM) of coated glass. (a) Coating 2 and (b) Coating 5.

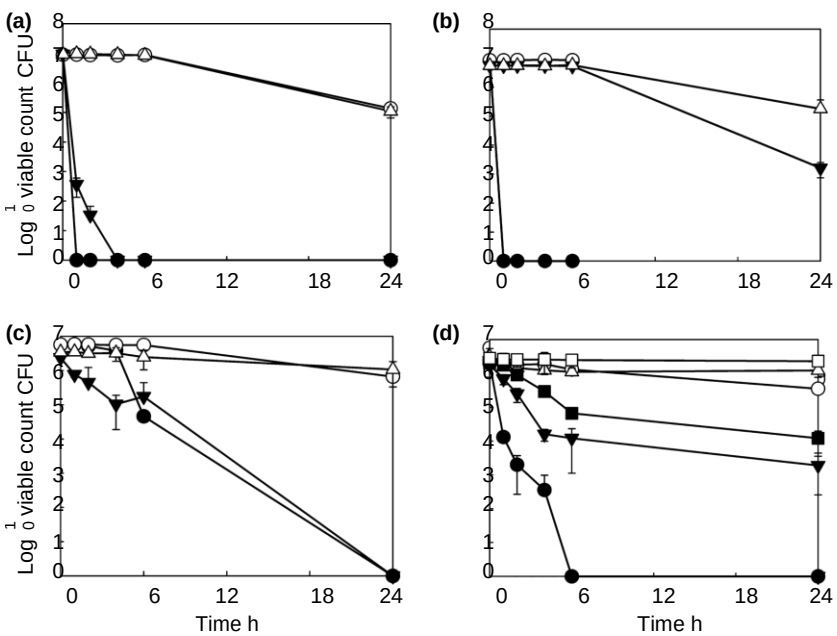

Figure 3 Antibacterial activity of Ag-SiO_2 coating 2 against test bacteria. (a) (●) Escherichia coli ATCC8739 test; (◯) E. coli ATCC8739 control; (▼) Staphylococcus aureus ATCC9538 test; (▽) Staph. aureus ATCC9538 control. (b) (●) Pseudomonas aeruginosa NCIMB 10421 test; (◯) Ps. aeruginosa NCIMB control; (▼) Ps. aeruginosa AOH1 test; (▽) Ps. aeruginosa AOH1 control. Antibacterial activity of Ag-SiO_2 coating 5 against hospital isolates. (c) (●) Enterococcus faecalis test, (◯) Ent. faecalis control, (▼) MRSA (NCTC 12493) test, (▽) MRSA (NCTC 12493) control, (d) (▼) Acinetobacter baumannii test, (▽) Ac. baumannii control, (■) Stenotrophomonas maltophilia test, (□) Sten. maltophilia control, (●) Klebsiella pneumoniae test, (◯) Kl. pneumoniae control.

reduction up to 6 h but a $\log_{10}$ reduction factor of 1 after 24 h.

The new coater head was used for coating both glass (coating 5) and tiles (coating 6). The standard E. coli strain was reduced by a $\log_{10}$ factor of >5 within 1 h on coating 5, the same as coating 2 (not shown). The MRSA strain was much more resistant than the MSSA, but there was still a $\log_{10}$ reduction factor of >5 after 24 h (Fig. 3c). The Ent. faecalis also had a $\log_{10}$ reduction factor of 2 after 6 h increasing to >5 after 24 h Fig. 3c). The activity of coating 5 against Ac. baumannii, Kl. pneu-moniae and Sten. maltophilia is shown in Fig. 3d. Steno-trophomonas was most resistant with a $\log_{10}$ reduction factor of 1 after 4 h increasing to 2 3 after 24 h, whereas Acinetobacter had a $\log_{10}$ reduction factor of 2 after 4 h increasing to 3 after 24 h. Klebsiella pneumoniae was the most sensitive of the hospital isolates but was slightly more resistant than E. coli and had a $\log_{10}$ reduction factor of >5 after 6 h (Fig. 3d).

The effects of protein on the activity of the coatings against E. coli are shown in Fig. 4. The addition of even 10 g l^{1} BSA to cells suspended on coating 2 completely inhibited killing, and no killing was seen after 2 h even though the controls were killed within 45 min (Fig. 4a). Increasing the amount of Ag in the film (coating 3) gave some killing with 10 g l^{1} protein ($\log_{10}$ reduction factor of 2), but activity was completely inhibited at 50 and 100 g l^{1} protein (Fig. 4b). Increasing the Ag content further (coating 4) gave a $\log_{10}$ reduction factor of >5 after 24 h with 10 g l^{1} protein and a $\log_{10}$ reduction of three with 50 g l^{1} protein (Fig. 4c).

The effects of ageing and washing on coating 2 are shown in Fig. 4d. The ageing cycle (samples irradiated with UV and exposed to vapour at 70°C) reduced activity against E. coli but still gave a $\log_{10}$ reduction factor of >5 after 6 h. Putting the samples through the washing cycle also reduced the activity, but a > 5 $\log_{10}$ killing was still obtained after 4 h (Fig. 4d).

The placement of the tiles on the wooden board and the placement in the ladies toilet facility are shown in Fig. 5. This was part of a larger study evaluating the per-formance of a number of coatings on glass, ceramic tiles and steel, the results of which will be reported elsewhere. The recovery of bacteria from the Ag-SiO_2 tiles by swab-bing is shown in Fig. 6. The coated tiles had a 95% lower surface contamination than the control tiles after 2 weeks and 99 8% lower after 4 months. The increase in overall contamination between months 3 and 4 can be ascribed to increased use of the toilets following return of the university students after the summer vacation.

Discussion

The results show that the durability of the coatings depended on the amount of Ag in the film. Films with lower Ag content had a Mohs hardness of >5 equivalent

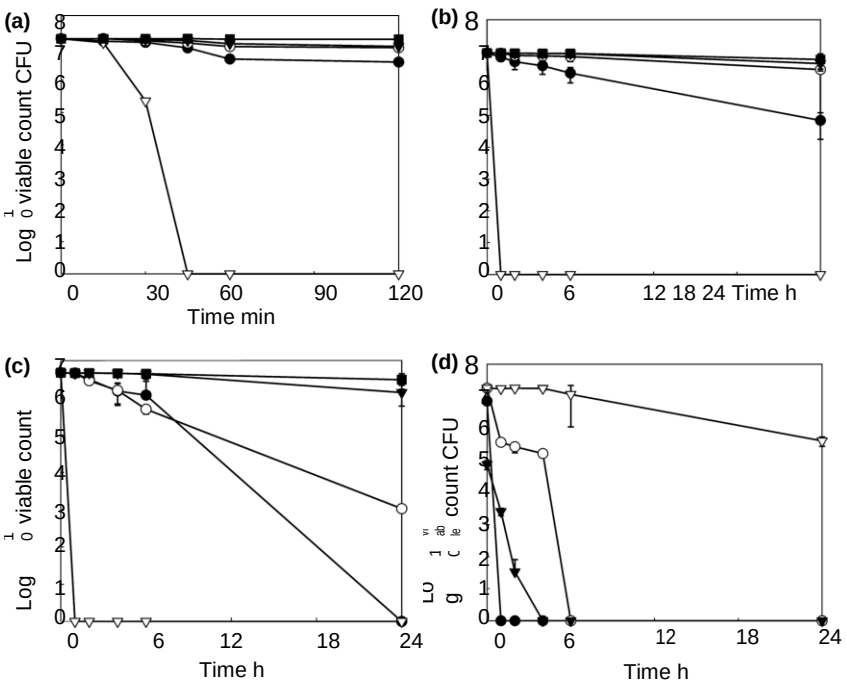

Figure 4 Effects of protein and washing/ ageing on antimicrobial activity against Escherichia coli 8739. (a) Coating 1, (b) Coating 3 and (c) Coating 4. (●) 10 gl ^{1} BSA, (○) 5 gl ^{1} BSA, (▼) 100 gl ^{1} BSA, (▽) No protein, (■) control. (d) Effects of ageing and washing on coating 2. (●) Untreated control, (○) Aged, (▼) Washed, (▽) Uncoated control.

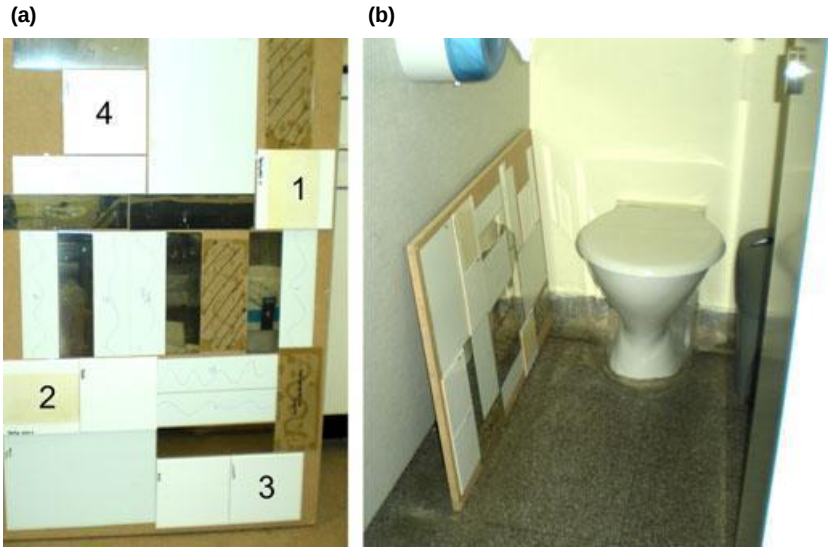

Figure 5 In situ testing of Ag-SiO_2-coated ceramic tiles. (a) Arrangement of tiles on test board. (b) Placement of board in toilet. 1 and 2 test tiles, 3 and 4 control tiles.

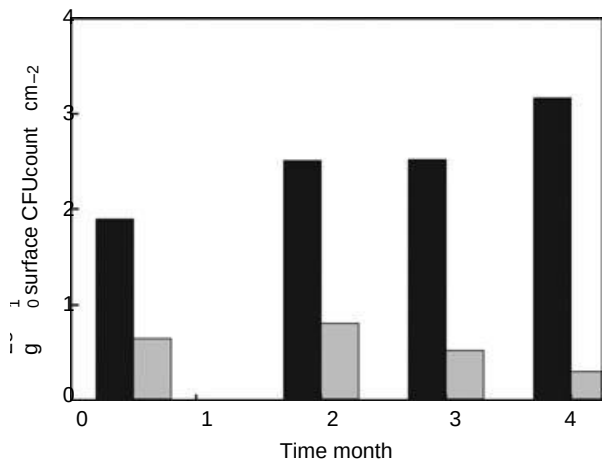

Figure 6 Antibacterial activity of Ag-SiO_2 coating 5 on ceramic tiles placed in ladies toilet facility. (■) Control; (▯) Test

to steel and were well adhered to the substrate but increasing the amount of silver reduced both hardness and adhesion. The results also show that the Ag-SiO_2 coatings had good activity against standard test strains of bacteria with a $\log_{10}$ reduction factor of >5 after 1 h for Gram-negative bacteria and 6–24 h for Gram-positive bacteria depending on strain and Ag content of the film. The strains tested were all as specified for disinfectant testing and show that the activity against other isolates, including a wild isolate of Ps. aeruginosa, MRSA, Ent. faecalis and ESBL-producing Gram-negative bacteria was lower although still giving a minimum 99% reduc-tion after 24 h. The BS test has several drawbacks. Firstly, the level of contamination is very high (approx. 10^6 CFU cm ^{2}) to allow the detection of the killing of the test organisms. This is much higher than levels of environmental contamination that have been measured in some outbreaks (<10^2 CFU cm ^{2}, Otter et al. 2011). A 99% reduction would reduce this to <1 CFU cm ^{2}. Secondly, the use of a bacterial suspension will allow diffusion of the antimicrobial (Ag or Ag^+ in this case) from the surface; this effect would be reduced if the surface contamination was dry or the suspension dried quickly. Evaluation of activity using air-dried deposits may be more realistic for comparison with in situ contamination. There is clearly a need for further standardized tests that more closely mimic the in use conditions. The increased resistance of the hospital and wild isolates compared with the disinfectant test strains shows that the latter should also be used for the evaluation of potential in situ perfor-mance of antimicrobial coatings. In an attempt to evalu-ate the in situ performance of the tiles, samples were placed in a toilet facility where the tiles were subjected to natural contamination. The coated tiles performed well for the first 4 months giving 95–99% reduction in surface contamination compared with control tiles.

Silver binds to a number of components including the sulfhydryl groups of amino acids in proteins (Liau et al. 1997). We therefore examined the effects of protein as an interfering agent on killing activity against E. coli to mimic the effects of contamination with, for example, body fluids. The presence of even 10 g l ^{1} BSA completely inhibited the antimicrobial activity on coating 2 containing low amounts of Ag. The killing effect could be increased by increasing the amount of Ag, and coating 4 gave a $\log_{10}$ reduction of >5 after 24 h (cf 1 h without protein). These experiments were performed to mimic the effects of contamination with, for example, serum, which may occur in use and which has a total protein content of approx. 80 g ^{1} which would reduce the activ-ity of coatings with a lower Ag content. Increasing the Ag content of the coating reduced the inhibition of antimicrobial activity by protein. However, increasing the amount of Ag also decreased the hardness and adhesion of the film (see also Cook et al. 2011). It is likely that in use, any contamination with blood, etc.,

would be removed by cleaning. The resistance of the coatings to cleaning shown here indicates that the activity can be retained although long-term tests of durability will be required.

Previous studies using a silver-based persistent disinfec-tant gave a $\log_{10}$ reduction factor of 3 in 10 min (Brady et al. 2003). We have also observed rapid killing of E. coli on surfaces electrochemically coated with Ag (Alex Steele and Howard A. Foster, unpublished data). The speed of killing seen here is slower than on the electrochemically coated surfaces, and this is probably due to the lower amount of Ag in the CVD coating and slower release from the surface. The rate of killing on glass surfaces with Ag nanoparticles has been shown to be related to the release of Ag from the surface (Esteban-Tejeda et al. 2012). Furthermore, the rate of killing we observed was similar to the killing of Legionella pneumophila and Staph. aureus on Ag/Zn-zeolite coatings on steel (Bright et al. 2002; Rusin et al. 2003) or added to high impact polystyrene coating on steel refrigerator linings (Kamp-mann et al. 2008), which also release the Ag slowly. The results suggest that the coatings produced here may be useful for surfaces in hospitals and other areas where control of micro-organisms is important, for example, in food manufacture although the amount of Ag in the film will need to be a balance between activity and durability. The Ag-SiO_2 coatings can be very hard and durable and the thinness of the coating reduces the cost compared with thick coatings. These surfaces will not replace clean-ing and disinfection but will provide a continuous anti-microbial activity in periods between implementation of such measures.

Acknowledgements

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Conflict of interest

No conflict of interest declared.

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Antimicrobial activity of novel nanostructured Cu-SiO_2 coatings prepared by chemical vapour deposition against hospital related pathogens

Sajnu Varghese[1], Souad O ElFakhri[1], David W Sheel[2,3], Paul Sheel[3], Frederick J Eric Bolton[4] and Howard A Foster[1*]

Abstract

There is increasing recognition that the healthcare environment acts as an important reservoir for transmission of healthcare acquired infections (HCAI). One method of reducing environmental contamination would be use of antimicrobial materials. The antimicrobial activity of thin silica-copper films prepared by chemical vapour deposition was evaluated against standard strains of bacteria used for disinfectant testing and bacteria of current interest in HCAI. The structure of the coatings was determined using Scanning Electron Microscopy and their hardness and adhesion to the substrate determined. Antimicrobial activity was tested using a method based on BS ISO 22196:2007. The coatings had a pale green-brown colour and had a similar hardness to steel. SEM showed nano-structured aggregates of Cu within a silica matrix. A log10 reduction in viability of >5 could be obtained within 4 h for the disinfectant test strains and within 6 h for producing Acinetobacter baumannii, Klebsiella pneumoniae and Stenotrophomonas maltophilia. Activity against the other hospital isolates was slower but still gave log10 reduction factors of >5 for extended spectrum β-lactamase producing Escherichia coli and >3 for vancomycin resistant Enterococcus faecium, methicillin resistant Staphylococcus aureus and Pseudomonas aeruginosa within 24 h. The results demonstrate the importance of testing antimicrobial materials destined for healthcare use against isolates of current interest in hospitals as well as standard test strains. The coatings used here can also be applied to substrates such as metals and ceramics and have potential applications where reduction of microbial environmental contamination is desirable.

Keywords: Antimicrobial; Chemical vapour deposition; Copper; Disinfection surface; Hospital pathogens

Introduction

It is now accepted that environmental contamination plays a key role in the transmission of infectious diseases in the healthcare setting (Hota, 2004; Boyce, 2007; Bartley and Olmsted, 2008; Dancer, 2009; Weber et al., 2010; Otter et al., 2011). There is a higher risk of acquiring a HCAI if the previous room occupant had such an infec-tion (Shaughnessy et al., 2011). Pathogens can survive on surfaces for prolonged periods of time depending on the organism and environmental conditions (Kramer et al., 2006; Neely and Maley, 2000; Wagenvoort et al., 2011) and can be transmitted to hands from the environment (Bhalla et al., 2004). Enhanced environmental cleaning has been shown to reduce rates of infection (Dancer et al., 2009; Kochar et al., 2009; Carling et al., 2010; Kleypas et al. 2011). However, the environment rapidly becomes recon-taminated following disinfection (Hardy et al., 2007).

One possible approach to controlling environmental contamination has been the reintroduction of copper (Cu) into hospitals (Sasahara et al., 2007; Casey et al., 2010; Mikolay et al., 2010; Espirito Santo et al., 2011; Schmidt et al., 2012) and other healthcare settings (Marais et al. 2010). Cu is widely used for its antimicrobial properties (Borkow and Gabbay, 2009; Grass et al., 2011). Cu and Cu alloy surfaces have been shown to kill a variety of patho-gens including Salmonella enterica and Campylobacter jejeuni (Faundez et al., 2004), Listeria monocytogenes (Wilks et al., 2006), methicillin resistant Staphylococcus aureus (MRSA; Noyce et al., 2006a; Gould et al., 2009; Michels et al., 2009; Weaver et al., 2010), Escherichia coli O157 (Wilks et al., 2005; Noyce et al., 2006b), Mycobacter-ium tuberculosis (Mehtar et al., 2008), Clostridium difficile

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(Wheeldon et al., 2008; Weaver et al., 2008), Pseudomonas aeruginosa (Gould et al., 2009) and enterococci (Gould et al., 2009; Warnes and Keevil, 2011). A crossover study compared conventional surfaces for a toilet seat, tap han-dles and a ward entrance door push plate with Cu con-taining items (Casey et al., 2010). The study showed reduced (>90%) bacterial counts on the items containing Cu and no indicator pathogens (e.g. VRE) were isolated during the 5 week study period on the copper whereas they were detected on control surfaces. A further longer study in an intensive care unit showed a sustained reduc-tion of 83% in microbial surface counts on six commonly touched Cu containing surfaces and numbers on control surfaces were also reduced (Schmidt et al., 2012). This was accompanied by a reduction in the rates of infection on the unit (Salgado et al., 2013). Recolonisation of Cu surfaces following cleaning has been shown to be delayed compared to control surfaces (Mikolay et al., 2010). However, Cu surfaces may become conditioned allowing colonisation following cleaning (Airey and Verran, 2007) possibly by retention and survival of organisms in surface scratches (Verran et al., 2010).

Sol–gel methods can be used to producing durable Cu containing glasses but these have not been investigated for their antimicrobial properties (Perez-Robles et al., 1999; Tohidi et al., 2010). Coatings with antimicrobial activity containing Cu nanoparticles have been prepared by surface coating with a powder mixture followed by heat treatment to fuse the particles into a glass-like coat-ing (Esteban-Tejeda et al., 2012). In the present study chemical vapour deposition (CVD) was used to produce thin film coatings. In this process a reactive gas mixture containing coating precursors is introduced into a coat-ing region and a source of energy e.g. heat applied to ini-tiate (or accelerate) decomposition of the precursor and growth of the coating on the target substrate (Choy, 2003). Atmospheric Pressure CVD (APCVD) has been widely used e.g. for production of self-cleaning coatings for glass which generally have excellent hardness and durability. We have previously reported the use of CVD to produce antimicrobial SiO_2 (silicon dioxide) coatings containing silver (Ag; Cook et al., 2011; Varghese et al., 2013). In this report we describe the activity of similar coatings but containing Cu instead of Ag as the anti-microbial component. The activity of these Cu-SiO_2 (copper-silicon dioxide) coatings against hospital related pathogens that are of current interest was investigated.

Materials and methods

Microorganisms and growth conditions

Escherichia coli ATCC 8739, and Staphylococcus aureus ATCC6538 were obtained from the American Type Cul-ture Collection. Pseudomonas aeruginosa 10421 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen U.K. and strain AOH1 was a wild isolate recovered from the river Tame downstream from Greenfield Wastewater Treatment Works. Extended spectrum β-lactamase (ESBL)$^+$ Acinetobacter baumannii, KPC$^+$ (carbapenemase) Klebsiella pneumoniae, ESBL$^+$ Escherichia coli, EMRSA15, two recent isolates of MRSA, MRSA 1599 and MRSA 1665, MRSA NCTC10492, Stenotrophomonas maltophilia, and vancomycin resist-ant Enterococcus faecium (VRE) were obtained from the Health Protection Agency, Manchester, U.K. and sub-cultured onto Nutrient Agar (NA, Oxoid, Basingstoke, UK) and incubated at 37°C for 24 h. Cultures were resuspended in Nutrient Broth (NB, Oxoid) and kept on Microban® beads (TCS Ltd Merseyside, UK) at −70°C. Prior to use, one bead was sub-cultured onto NA and incu-bated at 37°C for 24 h.

Production of coatings

Cu-SiO_2 coatings were deposited on 1 mm borosilicate glass (Dow Corning) using flame assisted chemical vapour deposition (FACVD). The FACVD system was of in-house design and construction and consisted of a brass burner head above a translational stage and a precursor delivery system of ultrasonic nebuliser, bubbler and mass flow con-trollers (Cook et al., 2011). Tetraethylorthosilicate was carried to the burner head using a nitrogen flow rate of 0.5 $lmin^{-1}$ from a heated and stirred bubbler (75°C ± 3°C, stirred at 120 rpm). An aqueous solution of copper sulphate (0.25 M) was used as the copper precursor and simultan-eously delivered to the burner head by ultrasonically nebulising the aqueous solution prior to carriage by nitro-gen at 0.6 $lmin^{-1}$. The number of passes under the burner head was 6 equating to a residence time in the flame of ap-proximately 12 sec and gave a film approx 25 nm thick.

In the later stages of the study a new coating head was used which was capable of coating 10 cm wide sub-strates. Copper content of the films was varied by chan-ging the concentration of the precursor and the flow rate to the coating head. The different conditions for the coatings are shown in Table 1.

Characterisation of coatings

To assess the hardness of the deposited coatings, films were scratch tested using a constant load scratch hard-ness tester. A diamond tipped scribe was moved through 50 mm over the surface with a 100 g load. The mean width of the resulting scratch over 6 points was then measured under 200× optical magnification and compared to similar data from materials of known Mohs hardness (aluminium, steel, copper, glass and quartz) and Mohs hardness values of the deposited films were calculated. Results are the means of three determinations.

Adhesion of the coating to the substrate was determined by Scotch tape testing. The coating was cross hatched

Table 1 Coating conditions used in this study and their physical characteristics

Coating number	$Cu(NO_3)_2$ precursor concentration M	Flow rate to burner head l min^{-1}	Mohs hardness	Transmission %[#]
1	0.25	0.6	5.6	90.7
2	0.25	2*	3.6	87.9

*Second coater head. [#]Control glass had 91.5% transmission.

every 5 mm with a diamond scribe, the adhesive tape was then applied and pressed firmly to ensure consistent contact with the coating. On removal the tape was observed visually and then under a microscope to determine if the integrity of the film had been maintained.

Appearance of the films

Transmission of the coatings in visible light was measured using an Aquilla NKD7000 spectrometer using plane polarized light source and transmission averaged over 400-700 nm and measured at a 30° angle.

Surface morphology was investigated using Scanning Electron Microscopy (SEM; Philips XL30) with samples sputter coated with a 2–3 nm layer of Pt/Pd to provide a conductive surface.

Testing for antimicrobial activity

Antimicrobial activity was tested based on BS ISO 22196:2007 (Anon. 2007) except that glass covers were used rather than plastic, the test was done at 20-25°C rather than 35°C (see discussion) and samples were tested after different times rather than just after 24 h as specified in the test. Twenty mm square samples of coated and control glass were sterilized by placing in 90% methanol for 20 min. The squares were transferred to a sterile Petri dish and left for at least 1 h to allow the methanol to evaporate. Colonies were resuspended in a 1:500 dilution of NB and adjusted to OD 0.01-0.02 at 600 nm in a spectrophotometer (Camspec, M330, Cambridge, UK) to give approx. 2×10^7 colony forming units (cfu) cm^{-3}. Fifty μl was inocu-lated on to each test sample and covered with an 18 mm square of 1 mm borosilicate glass to ensure close contact between the culture and the film. The samples were placed in 50 mm diameter Petri dishes containing moistened filter paper to prevent drying out of the suspensions. Plain boro-silicate glass was used for controls. Samples were removed after 0, 1, 2, 4, 6 and 24 h and immersed in 20 cm^3 of ster-ile Tryptone Soy broth (Oxoid) together with the cover glass and vortexed for 60 sec to resuspend the bacteria. A viability count was performed by dilution and plating on NA in triplicate and incubation at 37°C for up to 48 h. TSB had previously been shown to inactivate cop-per released from the surfaces at up to 1 mM by incuba-tion of cultures in TSB supplemented with $CuSO_4.5H_2O$ (data not shown).

In order to determine the effects of protein on antimicrobial activity, bovine serum albumen (BSA: Sigma-Aldrich, Poole, Dorset, UK) was added to the 1:500 NB used for production of the test culture suspensions be-fore inoculation onto test and control surfaces at a final concentration of 10 gl^{-1}.

Statistical analysis

Where possible each experiment was done in triplicate and means and standard deviations calculated using Microsoft Excel. Survival curves were plotted as the means with standard deviations as error bars. In order to allow plotting survival curves on a logarithmic scale, because zero cannot be plotted on a logarithmic scale, one was added to each mean viable count. In some cases error bars were obscured by the graph symbols and in others only upper error bars were plotted.

Results

The films had a pale brown-green tinge which was darker with the higher Cu content (Figure 1). Transmission in the visible range was 88-91% compared to 91.5% for the control glass (Table 1). The Cu incorporation reduced the transmission as expected especially with higher concentrations of Cu. SEM showed an amorphous background with evenly distributed aggregates which increased in size and number with higher concentrations of Cu (Figure 2).

The Mohs hardness of films were 5.6 for coating 1 and 3.6 for coating 2 giving a hardness comparison to stain-less steel for coating 1 (stainless steel = 5.5). For all films the coating remained intact in the Scotch tape test showing a good adhesion to the substrates. Coating 1 was used in most subsequent experiments, coating 2 was only used to study the effects of protein on antimicrobial activity.

The antimicrobial activity of coating 1 against the standard test strain of E. coli (ATCC8739) is shown in Figure 3a. There was a log10 reduction factor of >5 after 4 h. The ESBL producing E. coli was more resistant and

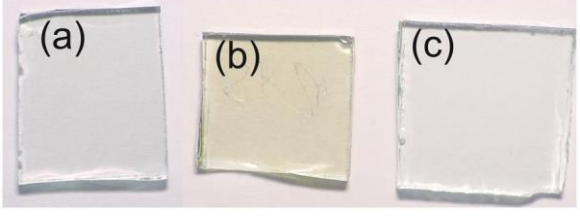

Figure 1 Visual appearance of coated glass. (a) Coating 1, (b) coating 2, (c) control glass.

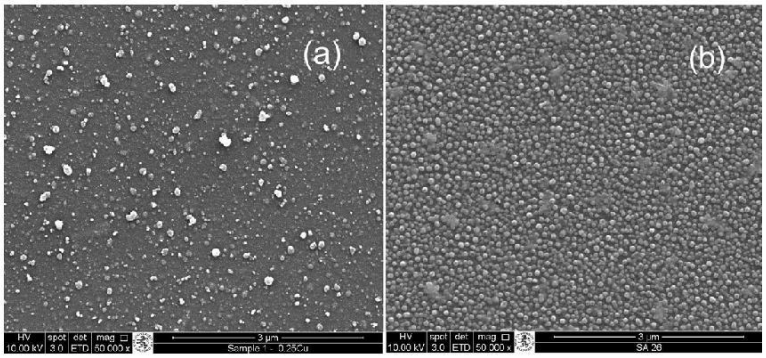

Figure 2 SEM analysis of CuO-SiO_2 coated glass. (a) coating 1 (b) Coating 2.

it took 24 h to obtain a similar reduction. The standard strain of P. aeruginosa was reduced by a log10 reduction factor of 5 after 6 h but the wild isolate AOH1 was only reduced by a factor of 3.5.

The activity against the Gram-negative clinical isolates is shown in Figure 4. ESBL producing A. baumannii and S. maltophilia (Figure 4a) and K. pneumoniae (Figure 4b) all had log10 reduction factors of >5 in 4–6 h. The activity of the coatings against Gram-positive organisms is shown in Figure 5. The disinfectant test strain of S.aureus (ATCC9538) was reduced by a log10 reduction factor of >5 after 6 h (Figure 5a). MRSA strains were more resistant with a log10 reduction factors of >5 for the type strain NCTC10492 after 24 h (Figure 5a) and approx. 3 after 24 h for EMRSA15 and the two recent clinical isolates MRSA1595 and 1669 (Figures 5a, b). The vancomycin resistant E. faecium gave a similar reduction (Figure 5b).

The effects of addition of protein on the antimicro-bial activity against E. coli ATCC9739 are shown in Figure 6. With coating 1 the activity was completely inhibited and there was no killing even after 24 h. With coating 2, which had a higher Cu content, the activity was slowed but there was a log10 reduction factor of >5 after 6 h.

Discussion

The structure of the films under SEM suggests that there are islands of Cu which extend above the surface of the film. This is consistent with our previous observa-tions of Ag containing CVD coatings where a clear is-land structure was observed for Ag only films and in Ag-SiO_2 composite films we identified embedded silver nano-particles which demonstrated a degree of crystal-linity (Cook et al., 2011). The scotch tape test and scratch testing showed that the coatings were well at-tached to the substrate and had abrasion resistance equivalent to steel. This is important in the resistance to wear during the expected lifetime of the products (10– 15 y). The benefit of using CVD is that good properties are achievable with a relatively simple process. Capital costs for equipment are also relatively low. These points are a significant advantage versus, for example, sputtering

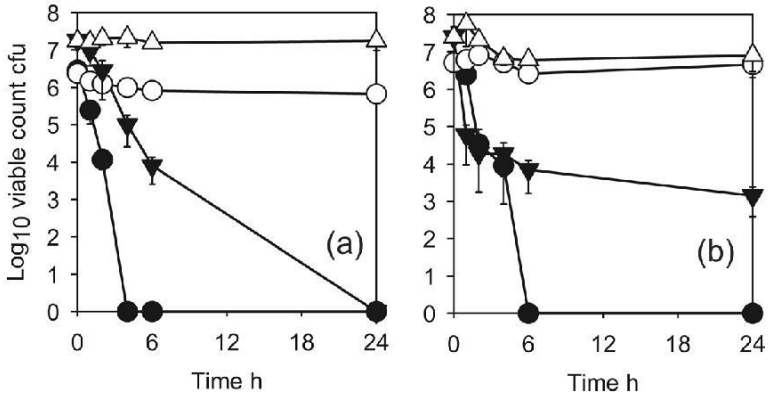

Figure 3 Antimicrobial activity of Cu-SiO_2 coating against Escherichia coli and Pseudomonas aeruginosa(d). (a) ● Escherichia coli ATCC8739 (disinfectant test strain) test; ○, ATCC8739 control; ▼, ESBL E. coli test; ▽, ESBL E. coli control ○. (b) ●; Pseudomonas aeruginosa 10421 (disinfectant test strain) test; ○, P. aeruginosa 10421 control, ▼, P. aeruginosa AOH1 (wild isolate) test; ▽, P. aeruginosa AOH1 control. Coating 1.

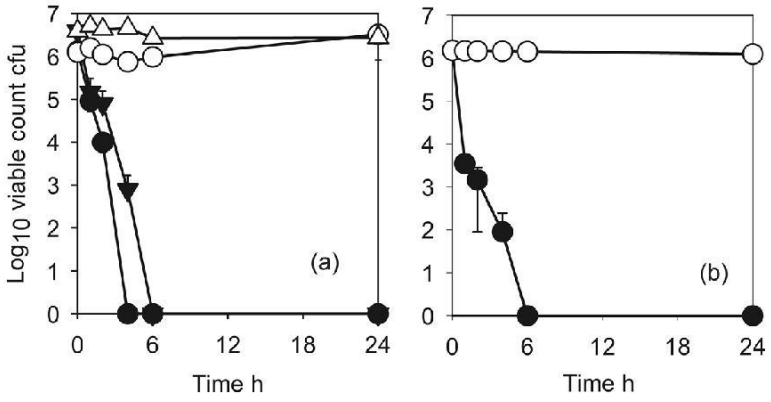

Figure 4 Antimicrobial activity of CuO-SiO_2 coating against Gram-negative pathogens. (a) ●, $ESBL^+$ Acinetobacter baumannii test; ◯, A.baumannii control; ▼Stenotrophomonas maltophilia test; ▽, S.maltophilia control. (b)●, KPC^+ Klebsiella pneumoniae test; ◯, K. pneumoniae control. Coating 1.

processes. Although we did consider some other ceramics (e.g. tungsten oxide, molybdenum oxide) we chose to use silica because we had done a lot of previous work on this matrix by FACVD (Cook et al., 2011, Varghese et al, 2013) and silica and silica precursors are compatible with FACVD.

The results show that organisms such as ESBL produ-cing K. pneumoniae and A. baumannii were killed rela-tively quickly on the Cu-SiO_2 coating. ESBL E. coli was more resistant than the standard test strain but was still effectively killed within 24 h. The MRSA strains were much more resistant than the Gram-negative bacteria and were also more resistant than the standard test strain of S. aureus or even than a type strain of MRSA used for testing methicillin resistance. A similar result was obtained for MRSA with titanium dioxide-copper coatings (Foster et al., 2012) and may reflect increased resistance of MRSA to killing by copper. It should be noted that the numbers or organisms used in the BS ISO method are much higher than have been reported in environmental contamination during outbreaks of MRSA (2.5×10^5 vs 500 cfu cm^{-2}; Otter et al., 2011) although in the latter case the organisms may not have been evenly distributed. The activity detected would still reduce the MRSA to less than 1 cfu cm^{-2} as suggested/recommended by Dancer (2011) within 6 h pro-vided that the activity determined here was maintained in real use situations. The other Gram-positive bacterium tested was a vancomycin resistant E. faecium strain (VRE). This proved as resistant to the CuO-SiO_2 coatings as the MRSA although the reduction was equivalent to a 99.8% reduction after 24 h. Preliminary tests with extended incu-bation showed a log 10 reduction of >5 after 48 h for MRSA and VRE. VRE have been shown to be capable of persisting in the environment for months and even years

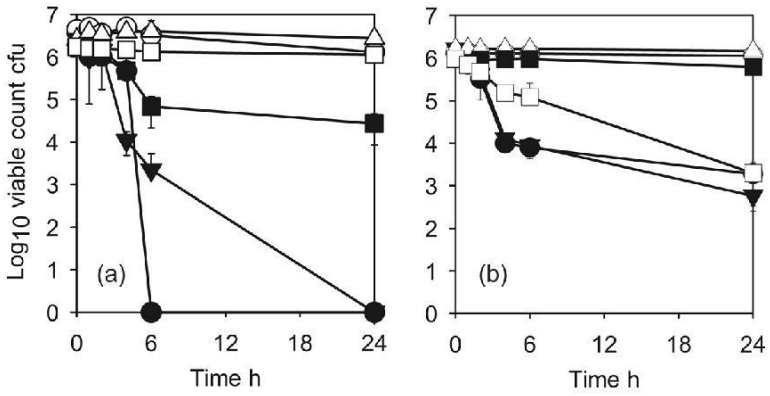

Figure 5 Antimicrobial activity of Cu-SiO_2 coating against Staphylococcus aureus and vancomycin resistant Enterococcus faecium. (a) ●, Staphylococcus aureus ATCC 9538 (methicillin sensitive disinfectant test strain) test; ◯, Staphylococcus aureus ATCC 9538 control; ■, EMRSA15 test; □, EMRSA15 control; ▼Staphylococcus aureus NCTC 12493 (reference MRSA) test; ▽ Staphylococcus aureus NCTC 12493 control. (b) □ Vancomycin resistant Enterococcus faecium test; ■, Vancomycin resistant E. faecium control; ◯, MRSA1599 test; ●, MRSA1599 control;▼ MRSA 1669 test; ▽, MRSA1669 control. Coating 1.

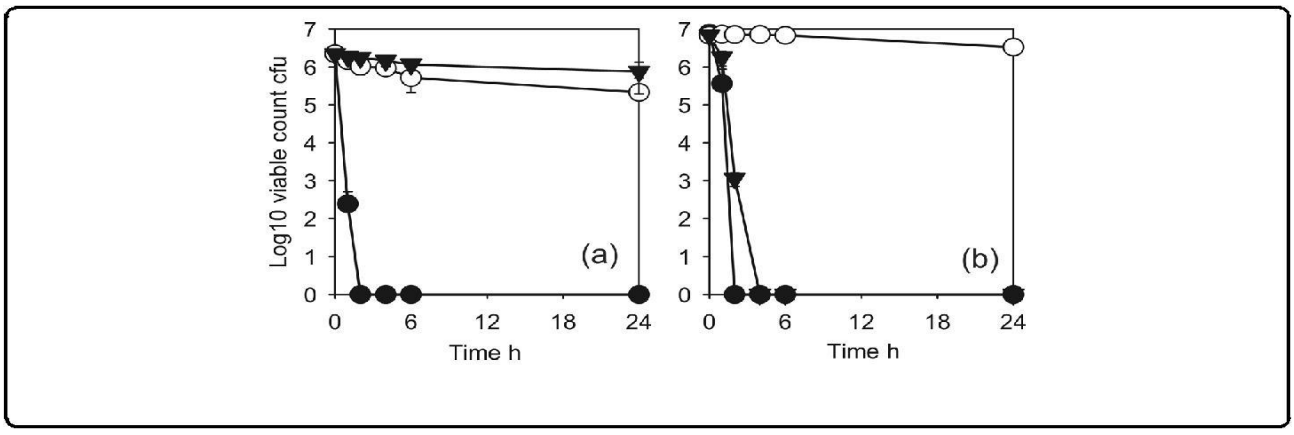

Figure 6 Effects of protein on antimicrobial activity of Cu-SiO_2 coating against Escherichia coli ATCC 10536. (a) Coating 1 ●, no protein; ▼, 10gl^{-1} bovine serum albumen; ◯, control. (b) coating 2 ●, No protein; ▼, 10gl^{-1} bovine serum albumen; ◯, control.

(Neely and Maley, 2000; Wagenvoort et al., 2011). The use of antimicrobial coatings in a healthcare setting should help to reduce this persistence.

Preliminary studies showed that Cu did elute from the Cu-SiO_2 coated surfaces (as Cu ions) and reached 20– 70 μM in the thin liquid films formed during the anti-microbial testing (determined by ICPMS, M. Abohtera and H.A. Foster unpublished). Relatively few studies report the bactericidal concentrations for Cu against bacteria and the value varies depending on medium composition and time of exposure. The minimum inhibitory concentration (MIC) for copper sulphate for several species of bacteria isolated from food animals was 20 mM for the majority of strains of E. coli and from 2–12 mM for S. aureus and Staphylococcus hyicus. Enterococcus spp. had a bimodal distribution with MIC from 2–24 mM (Aarestrup and Hasman 2004). Concentrations of eluted Cu^{2+} from the CVD coated surfaces are therefore much lower than MIC but there may be locally higher concentrations near to the surface of the coating. Alternatively, contact between the bacterium and the copper islands may allow diffusion of Cu^{2+} directly into the cell wall/membrane. Various mecha-nisms have been suggested for the antimicrobial activity of Cu including membrane damage, inhibition of respiration, protein inactivation and damage to DNA (Borkow and Gabbay, 2009; Grass et al., 2011). The mechanism may be different for bacteria with different cell-wall structure with membrane damage predominant in Gram-negative bac-teria and inhibition of respiration and DNA damage in Gram-positive bacteria (Warnes and Keevil, 2011, 2012). This may in part explain the differences in rates of killing seen here.

Interfering agents e.g. protein may be present in real use situations and even the low concentrations used here reduced the activity. Inhibition by amounts of protein that may be present in e.g. serum or food may be much higher. These surfaces will need to be cleaned to remove any such contamination but this is true of copper surfaces which can become conditioned in actual use (Airey and Verran, 2007). Increasing the amount of copper in the coating reduces the inhibitory effect of protein but the hardness of the coating is also reduced. The nature of the coatings used for in situ applications will need to be a com-promise between durability and activity.

We used a modification of the BS ISO 22196 method to allow determination of the rates of killing and we used room temperature to reflect activity at normal temperatures. Preliminary results suggested that activity was increased at higher temperatures giving an inflated impression of activity compared to room temperature. This method does not reflect the natural contamination that may occur in situ and there is an urgent need for standardised methods that reflect this as has been suggested by previous authors (O'Gorman and Humphreys 2012; Grass et al., 2011). However, the test does give information on the relative antimicrobial activity of the films against different organisms. A true test will be to determine the performance of the coatings in situ and also long term tests of durability and activity e.g. when subjected to washing and disinfection will need to be confirmed and these are currently under investigation.

The results suggest that application of the coatings to surfaces in the Healthcare setting may provide a useful background antimicrobial activity which functions continually and which may help reduce the inevitable recontamination which occurs following even the most penetrating disinfection treatments e.g. hydrogen perox-ide vapour fogging (Hardy et al., 2007). Environmental contamination has been shown to be important in trans-mission of a number of organisms including A. baumannii (Aygün et al., 2002) and VRE (Martinez et al., 2003; Hayden et al., 2008). The coatings may therefore have a role in reduction of transmission of such organisms in the healthcare setting, particularly as they can be applied

to different materials e.g. glass, ceramic tiles and metals. Indeed the surfaces that could be coated are only limited by the temperature reached during the coating process (approx 150°C) substrates would have to withstand this temperature. This will provide a range of materials for different uses which will be complementary to the use of copper and copper alloys and should contribute to the overall reduction in microbial contamination of the hos-pital environment with an associated reduction in trans-mission of infections. Used together with antimicrobial paints, fabrics, plastics and floor-coverings they may help to make the dream expressed by Bennett (2008) of a "self-disinfecting ward" a closer reality. These surfaces will not replace the normal cleaning and disinfection re-gimes but they will provide additional protection between such treatments. They may also find applications in other situations where control of microbial contamination is im-portant e.g. in the food industry. Increased use of copper may lead to an increase in copper resistance in bacteria. However, copper and copper alloys have been used for many years without widespread increases in the reported incidence of copper resistant pathogens.

Competing interests
CVD Technologies Ltd develop and install CVD coating equipment. The authors declare that they have no other competing interests.

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